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# A CUTANEOUS NEMATODE INFECTION IN MONKEYS.*

By HOMER F. SWIFT, M.D., RALPH H. BOOTS, M.D., and C. PHILIP MILLER, Jr., M.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

Plates 39 to 52.

(Received for publication, December 21, 1921.)

## INTRODUCTION.

While attempting to reproduce acute rheumatic fever in monkeys (*Macacus rhesus*), there were observed fever, subcutaneous nodules, and swellings about joints, which closely resembled some of the features of the disease in man. These features were first observed in monkeys inoculated with material from patients with acute rheumatism, but upon closer examination of the entire stock of animals, similar manifestations were occasionally encountered in normal animals and in some that had recovered from measles. In addition to subcutaneous nodules, the most striking lesion, upon casual observation, was a blister on the palms and soles, thought at first to be due to irritation from the disinfectant used in cleaning the cages. The keeper of the animal house stated that he had occasionally noted similar bullæ in monkeys imported in previous years. At first, no connection between these blisters and the subcutaneous nodules and periarticular edema was suspected, but later it seemed probable that all were dependent upon a single etiologic factor, a nematode. Search of the literature has failed to disclose a description of this disease in monkeys. As these animals are commonly used in laboratory experiments, it is thought advisable to make a note of the findings in order that other observers may be saved confusion.

### *Clinical Description.*

The subcutaneous nodules most closely resembling similar nodules in children or adolescents with acute rheumatic fever were found over

* Read before the Society for Experimental Pathology, New Haven, December 28, 1921.

the dorsal aspect of the hands and feet; they were easily missed unless the overlying hair was kept short by frequent shaving. The evolution of a single nodule was as follows: A circular area of subcutaneous edema from 4 to 10 mm. in diameter would appear. Careful palpation at times disclosed a small indistinct nodular thickening in the middle of the edematous area. After 2 or 3 days the edema would disappear and the nodule become more distinct. The smaller nodules, 1 or 2 mm. in diameter, were hard, globular, and definitely subcutaneous, so that the overlying skin could be easily moved over them; often they could be more readily detected by placing the hand or foot of the monkey between a light and the observer, when the overlying skin had a white or gray shining, translucent appearance. Larger nodules were not infrequently ovoid in outline and obviously more easily detected than the smaller ones (Fig. 1). Aside from edema, no objective signs of acute inflammation were grossly manifested in the skin or subcutaneous tissue; sometimes the animals gave evidence of moderate tenderness of the diseased area, at other times they seemed oblivious of any hurt. Following the initial appearance of the nodules they diminished in size so gradually that very little if any difference could be detected from day to day, but measurement every 3 or 4 days demonstrated a definite decrease. The smaller nodules persisted from 1 to 4 weeks; it was often difficult to determine definitely the exact day when they could no longer be detected. Small nodules of this type were seen most commonly over the dorsal aspects of the metacarpal or metatarsal bones, as if closely associated with the tendon sheaths in these regions; several were also seen over the metacarpophalangeal joints; and a few over the proximal phalanges. A few were found over the ulna just below the tip of the olecranon, and over the tendons in the lower third of the leg. Larger nodules, from 3 to 5 mm. in diameter, were occasionally found over the wrist or posterior aspect of the elbow joint. These larger nodules disappeared more slowly than the smaller ones; they never showed evidence of softening in the center.

A second type of subcutaneous lesion was found more frequently over the muscles of the arm (Figs. 2 and 3), forearm, and thigh, less frequently over the leg. It consisted of swellings of irregular size and extent, in which the process seemed to involve both the skin and sub-

cutaneous tissue. At times these papules were flat, from 5 to 10 mm. broad and from 8 to 20 mm. long, and seemed to be from 2 to 3 mm. in depth; at other times they were narrow ridge-like thickenings, 2 to 3 mm. broad and 5 to 30 mm. long, sometimes straight, but more often comma-, L-, or horseshoe-shaped. In some of these lesions indefinite nodules could be detected; in others the swelling assumed a diffuse serpiginous appearance. The nodules, if present, often did not have the definite discrete feeling on palpation that was so characteristic of the lesions over the tendons of the hands and feet. Occasionally, when the lesion was close to a superficially lying bony surface, discrete nodules were very distinct. The more diffuse appearance of many of the papular lesions over muscular areas seemed to be due to a more intimate association between the nodule and overlying skin, and possibly more persistent edema than was present in the lesions over tendon sheaths and joints. Occasionally larger single nodules similar to those about the joints were found over large muscle groups. Rarely, localized areas of firm cutaneous edema from 5 to 10 mm. in diameter were noted; they would persist from 2 to 5 days and quickly disappear without leaving any gross evidence of cellular infiltration. They resembled urticarial wheals more than subcutaneous rheumatic nodules.

A more extensive form of subcutaneous swelling was found about the ankles and wrists of some of the monkeys. It was usually seen in only one extremity at a time, although in one instance both feet were involved simultaneously (Figs. 4 to 7). It consisted of a diffuse thick, soft, edematous area, not sharply outlined; the overlying skin at times was glistening, due to the tension; but there was no other local sign of inflammation. Occasionally the monkey favored the affected limb. At times the edema was confined to the dorsum of the hand or foot (Fig. 8); again it was seen only about the internal or external aspect of the wrist or ankle joint. It lasted from 2 to 10 days. Once or twice the swelling disappeared after 2 or 3 days but reappeared after an interval of 3 to 5 days.

Attempts to obtain fluid by puncture of the swollen area yielded only a small amount of blood-tinged serum. Puncture of the underlying ankle joint in two instances yielded only 1 or 2 drops of normal joint fluid that gave no bacterial growth on ordinary aerobic cultures.

No nodules were found in the subcutaneous tissues following the subsidence of this massive edema; but it was soon found that this type of swelling was always associated with a peculiar blister of the palms or soles in the immediate neighborhood of the swelling. The nature of these blisters gave the clue to the causation of all of the lesions described.

The most obvious pathologic alteration of the palms or soles was an elongated blister 3 to 5 mm. broad, and 2 to 10 cm. long, serpiginous in form as though something had burrowed between the horny epidermis and corium and deposited an irritating substance in its tract. Closer observation of the evolution of the bulla revealed at first a small oval blister about 3 by 5 mm. in size, filled with a blood-tinged serous fluid (Fig. 9); a day or two later it increased to an elongated serpiginous blister filled with similar material (Fig. 10); after another day the fluid became purulent, and the lesions had a yellow color. At this time a portion of the epidermis would be knocked off and the contents of the blister discharged; at times the bulla burst before the fluid had become purulent. In either event, the epidermis over the blister usually desquamated, leaving a smooth elongated serpiginous area with borders composed of ragged skin (Fig. 11). Gradually the denuded areas were covered with normal skin; and after 2 or 3 months the hand or foot showed no evidence of having been diseased. With the exception of the integument at the base and side of the nails, the only cutaneous area that has shown the bullous type of reaction has been the horny skin over the palms and soles, and between the fingers and toes; occasionally a blister was seen at the border of the palms extending 1 or 2 mm. into the neighboring hairy skin.

Occasionally by examination of the palms or soles with a hand lens a very fine serpiginous burrow was seen under the epidermis; it was from 0.3 to 0.5 mm. wide and several centimeters long, gray to brown in color, with no gross evidence of inflammation of the tissue at its side. At times the end of the burrow seemed to communicate with a collapsed blister. It is possible that this fine lesion, appearing as though a thread had been drawn in an irregular direction under the epithelium, is the earliest manifestation of the disease in the palms and soles—a stage before any tissue reaction has occurred.

Examination of the fluid obtained from the blisters revealed a lemon-shaped egg, characteristic of certain nematodes. Later adult worms, the diameter of a hair, about 2 cm. long, were obtained from several lesions.

Examination of the stools failed to reveal either the worm or eggs of this type. Microscopic examination of the tissue removed during life indicated that all of the lesions were due to the presence of a worm.

*Temperature.*

The temperature of the monkeys varied from 101.5–103.5°F., with an average afternoon temperature of about 1°F. higher than that of the morning. The temperature of the room exerted a certain influence, the temperatures of the monkeys being relatively low when the animal house was cold and high when it was warm.

In general, the presence of blisters and nodules did not influence the temperature of the monkeys. One animal having nodules over long periods, and also inoculated with human joint fluid on two occasions, showed only an occasional rise in temperature above 103°F. In a few instances, when infection of the blisters had occurred, accompanied by swelling and redness of the ankle or wrist, there was fever of 105°F.

Certain of the monkeys, both control and inoculated, at various times had unexplained short febrile bouts.

*Blood Picture.*

The blood picture resulting from this nematode infection was difficult to determine, as most of the monkeys at this time harbored another nematode of the genus *Œsophagostomum*, this latter being found encysted in the wall of the intestine, mesentery, and omentum. In the monkeys examined, which showed blisters and nodules, the leucocytes varied from 13,000 to 30,000 per c.mm. Leucocyte counts of monkeys not showing such lesions varied from 14,000 to 20,000 per c.mm. Eosinophilia, 7 to 30 per cent, was present in all monkeys examined.

*Histopathology of the Subcutaneous Nodules.*

Though the various clinical pictures are better understood after a study of the histopathology of the nodules of different ages, it is preferable to give a composite description of the lesions of various ages rather than to describe single nodules. The worm will be described in detail later. In all of the subcutaneous lesions in which it was found, it was from 35 to 55 microns in diameter and delimited by a hyaline cuticle staining pink with eosin-methylene blue, red with Van Gieson, and pale red to grayish red with methyl green-pyronine. This cuticle was at times the only worm structure that could be detected in older nodules; it seemed to be the portion of the parasite most resistant to the defensive agencies of the host.

In the youngest lesion, in which very little except edema of the skin and underlying tissues was clinically demonstrable, the area upon removal had a diffuse, gray, moist appearance, and no definite nodule could be detected. Microscopic examination of such an area usually revealed a single cross-section of the worm in each section; the parasite was surrounded by a collar of epithelioid cells and polymorphonuclear eosinophils; the total thickness of the collar was from two to five cells (Figs. 12 and 13). Small blood vessels nearby showed a marked perivascular reaction made up of actively dividing epithelioid cells (endothelial leucocytes) and polymorphonuclear eosinophils; vessels situated at least 10 mm. from the worm often showed this perivascular reaction (Fig. 14). The surrounding connective tissue showed distinct edema with scattered eosinophils (Figs. 12 and 13). In some slightly older lesions the worm and collar of cells were surrounded by a narrow circular area filled with fibrin; this in turn was encompassed by an area made up of a loose network of fibrin and cells with branching cell bodies and pale vesicular nuclei (Fig. 14). Edema, perivascular reaction, and diffuse infiltration with eosinophils were present in the surrounding tissue of all of the early lesions. In an area slightly older than the one just described many newly formed thin walled blood vessels were seen; also a few polynuclear cells with two, four, eight, or more closely packed, pale vesicular nuclei and very little cell body; they were probably young giant cells.

In those lesions, however, in which a distinct nodule from 2 to 4 mm. in diameter was evident at the time of earliest observation, microscopic examination revealed several coils and cross-sections of the parasite in each section; it seemed as though the worm were confined to a smaller area with consequently a more intense local reaction (Figs. 15 and 16). Some coils of the worm were surrounded simply by coagulated blood, others by a collar of cells similar to that previously described; often many of these cells had deep staining pycnotic nuclei as if they had been injured by some toxic agent; still other sections of the parasite were surrounded by epithelioid cells, lymphocytes, eosinophils, and giant cells. In two nodules this area of worm, hemorrhage, and cell reaction was surrounded on all except one side, or peduncle, by a zone of fibrillar and granular material into which were growing many young actively dividing cells with pale, vesicular nuclei and cell bodies with long branching processes; these young cells could be seen arising from the connective tissue surrounding the nodule. This area of actively growing cells closely resembled the edge of a tissue culture (Fig. 16). The portion of the lesion called the peduncle was made up of many newly formed capillaries and cells with large, pale, vesicular nuclei. As one passed towards the normal connective tissue these cells approximated more and more the appearance of the fixed connective tissue elements. In none of the lesions of this age was there much capsule formation, although in some areas a mosaic of spindle cells with large pale nuclei could be detected; methyl green-pyronine stained sections showed plasma cells most numerous in this zone. Practically all of the small blood vessels within a radius of 3 to 5 mm. showed the marked perivascular reaction previously mentioned; about some of them many closely packed plasma cells were visible, and about others were numerous eosinophils. In fact, eosinophils were present throughout all portions of the sections, both in the zone of active cell proliferation and in the surrounding edematous tissue.

In a slightly older lesion the coils of worm were immediately surrounded by mononuclear cells in many of which the nuclei were deep staining and pycnotic; next to this zone there were a number of large giant cells mixed with many eosinophils and a moderate number of epithelioid cells (Figs. 17 and 18). Scattered throughout the nodule

were several areas made up largely of newly formed capillaries distended with blood. At the periphery of the nodule definite early capsule formation was present; outside of this the blood vessels still showed marked perivascular reaction, and small foci of edema were seen in the connective tissue in contradistinction to the diffuse edema seen in the earlier lesions. A nodule having approximately the same general structure showed in only eleven of the sections easily distinguishable cross-sections of the parasite; nearby were annular hyaline structures of the same thickness, diameter, and staining reaction as the cuticle of the worm; occasionally these rings were filled with mononuclear wandering cells. No large giant cells were seen in this lesion, but there were several smaller cells with eight to fifteen closely packed oval or round vesicular nuclei and a distinct cell body of a red, finely granular appearance in methyl green-pyronine stained sections. These cells closely resembled the large cells seen in the Aschoff bodies in the hearts of patients dying in the acute stages of rheumatic fever. In fact, in this nodule it would have been difficult to differentiate the histological structure from that of early subcutaneous nodules in children with rheumatic fever, had no sections of the worm been found.

In nodules 2 weeks or more old capsule formation was moremarked, many plasma cells were found immediately under the capsule, and young actively dividing cells were comparatively less numerous in the interior of the lesion. Distinct cross-sections of worms were found in only three out of seven old nodules examined; and in these only a few of the sections contained a single cross-section of worm. In one other lesion hyaline ring-like structures resembling the cuticular membrane were seen. In all of the older lesions containing a portion of the worm there were many large giant cells often surrounding the parasite like a ring; in nodules containing no worm they were less numerous and were absent in two nodules with very thick capsules (Fig. 19). It seems probable that these giant cells function to remove the parasite and disappear as soon as this task is completed; this view is substantiated by their being most numerous in the nodules in which the worm is undergoing disintegration and by their gradual disappearance in lesions in which no remains of the parasite can be found. The presence in the younger lesion of polynuclear cells with two, four,

eight, or more nuclei and the fact that these cells are not far from the worm suggest that the giant cells are formed from the young actively growing cells that have invaded the lesion. A study of many lesions of various ages revealed this giant cell development from cells containing two nuclei to old cells with many nuclei peripherally situated.

Nodules a month or more old consisted chiefly of fibrous tissue in which the cells were more densely arranged at the periphery (Fig. 19). Eventually these nodules completely disappear, for none could be found post mortem in several monkeys killed 3 or 4 months after the clinical disappearance of multiple subcutaneous lesions.[1]

*Histopathology of the Palmar and Plantar Lesions.*

The chief site of activity of the nematode in the palms and soles was in the epidermis, with only slight accompanying reaction in the superficial portion of the corium. The principal portion of the epidermis involved was the stratum mucosum below the stratum lucidum and above the stratum germinativum. An early lesion taken from a place where the worm was grossly detected, and cut so that several cross-sections of the parasite were present in a single section, showed different degrees of reaction. Occasionally a single cross-section of the worm was seen in the stratum mucosum of the epidermis with the neighboring epithelial cells appearing almost normal. In other places, close by, small areas slightly larger than the diameter of the parasite were seen filled with granular material, red blood cells, and degenerated epithelial cells (Fig. 20). Generally the area of reaction about the parasite was much larger and consisted

[1] Incidentally another type of subcutaneous nodule was found in several monkeys, most often about the extensor aspect of the elbows. Grossly they were 1 or 2 mm. in diameter and consisted of a small brown central portion surrounded by a tough white membrane. Microscopically the central portion was made up of six-sided cells with a thick membrane containing a more or less homogeneous material; the entire cell stained deep blue with methylene blue-eosin. They were thought to be vegetable cells making up possibly the points of thorns or splinters of wood. Surrounding the mass of vegetable cells were a few foreign body giant cells and a dense fibrous tissue capsule. Nodules of this type remained constant in size and never showed the perinodular and subcutaneous edema noted about the lesions containing a worm.

in cross-section of a vesicular area 1 mm. thick and 2 to 4 mm. in breadth. In early lesions before rupture of the blister, the worm was usually found close to the stratum germinativum, which in most places seemed to be intact, but occasionally small areas of this stratum were missing and the blister was in direct contact with the superficial layers of the corium. In these places it was not unusual to see capillaries coursing upward through the corium and emptying into the blister. In the superficial layers of the corium immediately under the blisters there was a definite increase of cells, usually mononuclear in type, but in places small groups of polymorphonuclear leucocytes were seen. Rarely did the small blood vessels of the corium show the perivascular reaction which was so prominent a feature in the subcutaneous nodules. Occasionally there was definite edema of the subcutaneous tissue surrounding the blister, with a moderate number of polymorphonuclear eosinophils scattered through the edematous area.

The contents of the early unbroken blister consisted of amorphous granular material, many red blood cells, and a few leucocytes, both mononuclears and polymorphonuclears; epithelial cells in various stages of degeneration were also present. In older blisters there were many polymorphonuclear leucocytes but fewer red blood cells and epithelial cells. In most of the unbroken blisters it was possible to find the characteristic eggs of the nematode, which in all instances contained fully developed embryos. At times, surrounding the eggs there was a thin, pink-staining, hyaline, annular structure, irregular in outline and two or three times the diameter of the egg. Some sections of the blisters contained the adult parasite, others did not; if present the number of cross-sections of the worm in a single section varied from one to six or eight. In some lesions excised following an unsuccessful attempt to remove the worm intact, small portions of the parasite were found winding through the epidermis. This peculiar winding made it difficult to obtain many complete uninjured specimens of the nematode from the blisters. Sections of skin removed after the blister had ruptured showed the stratum corneum and stratum lucidum separated from the stratum mucosum by an open space; usually the stratum germinativum was intact but occasionally it was missing for a short distance. The fact that the deepest layer of

epithelium was rarely completely destroyed and that there was little destruction of the superficial layers of the corium explains the usual absence of deep ulceration after the desquamation of the top of the blisters; the germinal layer of the epithelium was in condition quickly to repair the damage.

It is evident that the two types of lesions differ in more than one respect. In the subcutaneous nodule the nematode is smaller in diameter, and probably in length, although this point cannot be definitely determined because of the impossibility of obtaining specimens of the parasite except in sections from the subcutaneous tissue. The anatomy of the worm found in the nodules was much simpler than that of the female nematode found in the cutaneous blisters (Figs. 21 to 23). No bodies resembling eggs were ever seen in the worms in the subcutaneous lesions or in the tissue immediately surrounding them; on the other hand, eggs in various stages of development were found in all of the parasites in the epidermal blister, and eggs containing embryos were constantly present in the blisters. In both the subcutaneous lesion and the epidermis the first effect of the parasite seemed to be necrosis of the cells immediately surrounding it, followed by vesicle formation with a rapid filling of the vesicle by red blood cells.

The hemorrhage surrounding the female worm in the epidermal burrows and many of the worms in the subcutaneous tissue is probably due to some toxic substance secreted by this parasite. Schwartz (1) lately showed that certain nematodes contain substances that inhibit the coagulation of blood to a marked degree and appear to be similar to leech extract. This toxic substance is evidently of importance in insuring the worm a proper supply of food, for the adult female lives literally in a pool of blood. The finding of hemorrhage about many of the nematodes in the subcutaneous nodules is a point in favor of the theory that the worms in both the epidermis and subcutaneous nodules belong to the same species.

In the subcutaneous tissue the intensity of the local response was so great that the parasite was prevented from wandering farther; on the contrary it seems to have been quickly killed, firmly encapsulated, and subsequently removed like any other easily phagocytable foreign body. In other words, the response of the tissues of the host in this re-

gion was sufficient to prevent the parasite from continuing its existence. The conditions in the epidermal lesion, on the other hand, were morefavorable for the parasite. By continuing its existence in the epidermis during the egg-laying stage and constantly invading new skin areas, the female worm found favorable conditions for the deposition of its eggs and their extrusion into the external world where they might infect new hosts. The reaction in the epidermis never seemed to be sufficiently intense to kill the worm. On the contrary, the ease with which the blister burst and scattered its contents rendered especially easy the dissemination of the eggs. There is here a condition of almost perfect parasitism in which the injury inflicted by the nematode on the host is not sufficient to impair seriously the health of the host, but in which the local injury in the palms and soles is of such a nature as to favor the continuation of the existence of this peculiar nematode.

It has been impossible for us to determine the life history of this nematode. It is possible that some of the worms found in the subcutaneous lesions were males; but if they were males, one cannot state whether they migrated to the subcutaneous tissue after fertilizing the female or whether they reached this tissue before having completed their sexual function. We have examined post mortem several monkeys with active skin lesions and, with the exception of one lymph node containing a larval form and tubercle-like nodule, have found no lesions resembling the skin blisters or subcutaneous nodules in any of the viscera or muscles. In so far as our observations go they indicate that practically the entire lesion producing activity of this nematode is in the epidermis and subcutaneous tissue.

Attempts to hatch the eggs in moistened filter paper kept at body temperature for 2 months have failed to induce the embryos to migrate from the shells. The injection of the eggs into the skin of the palms and soles of monkeys and into the subcutaneous tissue of the extremities has not been followed by lesions like those observed in naturally infected animals; there was slight local reaction lasting 3 or 4 days, following which there was practically no gross evidence of the introduction of any irritating substance. The fact that fully developed embryos can be caused to emerge from the eggs obtained from skin blisters by simple mechanical pressure shows that they are probably in condition to continue their life's activity

immediately when brought under favorable conditions. It seems most probable that the digestive juices of the stomach or intestine act upon the opercular plug and allow the embryo to escape; it is well known that eggs of this character are usually hatched in the stomach or intestine, after which the embryos continue their activity by burrowing through the wall of the gastrointestinal tract and migrating to various parts of the body. An intermediary host is therefore not necessary for the continuation of this type of parasite.

We have searched in vain for the characteristic eggs in the feces of twenty monkeys that had shown skin lesions and in the entire intestinal contents removed post mortem from several other monkeys; Kofoid's (2) brine concentration method was employed to obtain the material for examination. The eggs of another common intestinal parasite, an *Œsophagostomum*, were found in the feces of most of the monkeys examined. It seems probable, therefore, that the only manner in which the *Trichosoma* eggs are extruded from the body of the monkeys is from the skin lesions. In so far as we are able to determine, this method of dissemination of eggs has not been previously described for nematodes.

It is not unusual to find lesions of the skin and subcutaneous tissue caused by animal parasites. Ground-itch is well known to be due to invasion of the skin by the larvæ of *Ancylostoma duodenale*. Looss (3) states that a similar condition may result from infection with larvæ of *Strongyloides stercoralis*. Subcutaneous tumors in man may be caused by *Filaria bancrofti*, *Loa loa*, *Agamofilaria georgiana*, and *Rhabditis niellyi*. The female guinea-worm (*Dracunculus medinensis*) burrows in the subcutaneous tissues and discharges young larvæ from her body, through an ulcer in the skin to the outer world. This is the closest parallel we can find in the literature to the action of our *Trichosoma* in which eggs are deposited in an epidermal blister before being discharged from the body of the host.

A somewhat similar microscopic lesion of the epidermis is seen in larva migrans, or creeping eruption, which is due to burrowing in the epidermis by the larvæ of a *Gastrophilus* (bot-fly). The gross picture, however, is different; it consists of a migratory red line in the skin from 1 to 9 mm. broad, in the center of which is a fine line due to the burrow. Minute vesicles are occasionally seen along the course of

the red line, but the entire lesion does not become bullous or purulent (4).

Castellani and Chalmers (5) report a rare skin disease of unknown etiology found in natives of Ceylon and South India; it is called dermatitis macrogyrata, and is characterized by the presence on the palms of the hands of one or two very large gyrations formed by scaling and crusty lesions. On removing the crusts and scales a broad shallow furrow can be seen situated in the epidermis. Neither fly larvæ nor fungi are found in the lesions. The photograph of the condition in Castellani and Chalmers' book resembles very closely the lesions seen in our monkeys after the removal of the top of the blister. It is interesting to conjecture upon the possibility of the two conditions being due to a common cause, inasmuch as *Macacus rhesus* comes from the same part of the world as the patients in whom dermatitis macrogyrata is found.

### *Description of the Nematode.*

*Female.*—Only a few intact specimens of the female worm were obtained from the blisters on the hands or feet. Many broken specimens were secured, all of which contained eggs. The worms were all of practically the same size; the head and esophagus were of about one-half the diameter of the portion of the body containing eggs; the body gradually increased in diameter from immediately behind the head to the tail. Measurements were as follows:

Head: Length 42 microns; width 52 microns.
Diameter of worm at posterior end of esophagus: 100 to 110 microns.
Diameter of posterior portion of body: 200 microns.
Length of body: 22 to 24 mm.
Esophageal portion of body: One-sixth of the entire length of the worm.

In unstained specimens fixed in alcohol and mounted in glycerol, the nematode showed a hyaline cuticle with slight annular striation. Beginning about the middle of the body were seen numerous highly refractile fine papillæ, arranged in two broad bands towards the posterior portion of the body. The cuticle about the head was about twice the thickness of that elsewhere; the head was thus slightly ovoid in shape. No teeth could be detected. The esophagus con-

sisted of a single chain of from 85 to 95 cells with the lumen in the center. The digestive tube then showed a slight sacular dilatation, followed by a long intestine which was arranged in loose coils in the posterior half of the body. The anus was terminal. The posterior end of the body was bluntly conical.

The vulva was situated just posterior to the termination of the cuboidal esophageal cells. There was a single ovary at the posterior end of the body. Between the ovary and the vulva the eggs could be seen in the uterus gradually developing into definite embryos and taking on the characteristic brown, lemon-shaped shell with round canal at each end covered by an opercular plug.

The eggs measured from 40 to 42 microns in breadth and 67 to 70 microns in length, had a thick highly refractile hyaline shell, usually brown in color, and contained a coiled embryo, which upon pressure could be caused to emerge through the opening at one end (Fig. 24).

The embryo had bluntly rounded ends and was filled with a uniform colorless material containing a group of highly refractile droplets toward the posterior end.

In sections of the epidermis stained with eosin-methylene blue, the female parasite was surrounded by a pink hyaline cuticle. Inside of this were two single rows of blue-staining cuboidal muscle cells; each row occupied about one-third the circumference of the worm (Figs. 25 and 26). Between these rows of cells and slightly overlapping the ends were more loosely arranged pink-staining cells with long communicating processes, considered as nerve cells. In all cross-sections a flattened annular structure, evidently the intestine, was seen lined with a single layer of cuboidal cells. In a number of instances the lumen of the intestine contained a few erythrocytes.

In some sections the remainder of the body was filled with a single cross-section of the uterus with maturing eggs (Fig. 26). Elsewhere, there were two or three cross-sections of the generative organs. Some contained young eggs, others consisted of a thin hyaline ring enclosing numerous long flat deep blue-staining bodies radially arranged and usually pointing towards the center. Mesial to these, there was a loose fibrillar meshwork with a few similar elongated bodies. In a few sections large cells resembling young eggs were seen in the

center of this organ, and in several longitudinal sections this organ (supposed to be the receptaculum seminis) was seen to communicate directly with the egg-containing structure (Figs. 25 and 27).

No sections were obtained through the esophagus of the female worm. In all of the sections above described the diameter of the worm was from 140 to 160 microns.

*Worm in Subcutaneous Nodules.*—In one series of sections the entire worm was included (Fig. 28). It was fairly uniform in size, and measured from 35 to 45 microns in diameter. It was surrounded by a thin hyaline pink-staining cuticle, inside of which was a single ring of cuboidal cells. In some places this ring of cells was almost continuous; elsewhere, it was broken into two segments each occupying about one-third the circumference of the worm, with one or two larger cells between the ends of the segments. In many cross-sections the remainder of the worm was filled with granular amorphous pink-staining material. On longitudinal section, one end of the worm was made up of cuboidal cells filling the entire parasite. These resembled the esophageal cells seen in the female, and evidently corresponded to the same pink-staining areas described in cross-sections. In other sections, most often towards the posterior end of the worm, were seen two circular structures made up of elongated flat deep blue-staining bodies similar in shape and size to those seen in the receptaculum seminis of the female worm. In longitudinal sections these flattened bodies were pointed anteroposteriorly. No lumen or structure resembling an intestine was seen in this specimen.

A fresh serpiginous lesion after excision showed on gross examination a small waxy line. In these sections many cross-sections of a worm were seen; they were larger and the worm was evidently more mature than the one described above; measurements of several cross-sections showed a diameter of about 55 microns (Figs. 29 to 31). There was a pink hyaline cuticle inclosing a single ring of cells. This ring was divided into four segments, two of which contained cells with rounded deep blue-staining nuclei, each segment occupying about one-third the circumference of the worm; the other two were made up of pink-staining cells resembling the nerve cells of the female worm. Back of the esophagus were two distinct structures in the body of the

worm. One was an elliptical ring lined with a single layer of flat cells, evidently the intestine. The other was a round body occupying about one-half the diameter of the worm and in places filled with small cells with solid deep blue nuclei; in other places the nuclei were arranged concentrically about a small lumen; in still other sections the lumen was larger and surrounded by a single layer of cuboidal cells. At the head end of the worm longitudinal sections showed large cuboidal esophageal cells, and cross-sections showed these cells occupying the entire diameter of the parasite inside of the muscle and nerve cells.

It was thought probable that this was a male worm. The muscle and nerve cells, esophageal cells, and intestine resembled similar structures of gravid female worms in the epidermis. The genital tract, however, was different.

The first of the worms described in the subcutaneous tissue was evidently a larval form, as neither the intestinal nor genital tract was well differentiated. The second was more mature, with a genital tract distinctly different from that of the adult female. Both forms were seen in other sections. It seems plausible, therefore, to conclude that the subcutaneous nodules contained both immature larvæ and fairly well developed male worms.

### *Classification of the Nematode.*

The parasite is clearly a member of the nematode superfamily Trichinelloidea Hall, 1896*a*, family Trichinellidæ Stiles and Crane, 1910. Hall (6) divides this family as follows:

"1. Male with 1 spicule or at least with a copulatory sheath .....Trichurinæ.
"Male without spicule or copulatory sheath.....2.
"2. Eggs spherical without true egg shell; ovoviviparous; males not parasitic in females; adult worms in digestive tract.....Trichinellinæ.
"Eggs variable in shape and with true, thick shell and opercular plugs; oviparous; males parasitic in the uterus of females; worms in kidney pelvis ureters or in the urinary bladder.....Trichosomoidinæ."

On characters of the female, namely size and shape of body and location in host, our worm can be safely eliminated from the subfamily Trichinellinæ. As an examination of ten gravid females failed

to reveal the presence of males in the uterus, this worm is apparently to be eliminated from the subfamily Trichosomoidinæ. Hall (1916*a*, p. 19) divides the remaining subfamily, Trichurinæ, as follows:

"1. Anterior esophageal region of body very slender and longer than the posterior portion, which is much thicker and contains the reproductive organs.....*Trichuris*.
"Anterior esophageal portion of body shorter than, rarely equal to, the posterior portion, which is only slightly thicker.....2.
"2. Worms with spicule; in digestive tract or urinary bladder, ducts, etc. .....*Capillaria*.
"Worms without spicule; in liver.....*Hepaticola*."

This key clearly eliminates the genes *Trichuris* from consideration. There remain for consideration the following closely allied genera: *Capillaria* Zeder, 1800*a*, and *Hepaticola* Hall, 1916*a*. *Capillaria*, *s.l.* (synonym *Trichosoma*) is divided by various authors into *Capillaria*, *s. str.*, *Thominx* Dujardin, 1845*a*, and *Calodium* Dujardin, 1845*a*, which are variously recognized as genera or as subgenera.

It will be noticed that to classify a species of Trichurinæ in its proper genus and subgenus, it is absolutely essential to examine the male worm, and this sex we have thus far been unable to find. Any generic classification is, therefore, only provisional and will of necessity be subject to possible change as soon as the male is studied.

The old collective genus of this group is *Trichosoma* Rudolphi, 1819*a*, which is *Capillaria* Zeder, 1800*a*, renamed. If we classify the worm in *Capillaria*, *s. str.*, *Hepaticola*, *Thominx*, or *Calodium*, it is clear that we predicate certain anatomical characters upon the basis of which the modern classification is founded; accordingly, we introduce an element of confusion in anatomy. By placing the worm in the collective genus *Trichosoma* (which is *Capillaria sensu lato*) we avoid all complications as to detailed points in anatomy, but make it inevitable that later the worm will be transferred to one of the modern and more restricted generic groups. Both courses are open to criticism, but it seems that the latter course is the more conservative, since changes of names are more easily made than changes in erroneous conceptions as to anatomy; further, *Trichosoma* gives the approximate, though not the taxonomically exact, location of the worm. Accordingly, the parasite is provisionally referred to *Trichosoma* pending the finding of the male.

As it has been impossible to find any reference to a dermal trichosome in monkeys, we assume that the species is new and we propose for it the name *Trichosoma cutaneum*, 1922.[2]

SUMMARY.

A number of monkeys (*Macacus rhesus*) were found to be infected with a nematode which gave rise to several types of skin lesions, subcutaneous nodules, edema about the joints, and elongated serpiginous blisters of the palms and soles.

In the subcutaneous nodules were found larval forms of the nematode and possibly adult male forms.

The reaction about these worms consisted of proliferation of fixed cells, and invasion of eosinophils, with subsequent presence of giant cells, young blood vessels, and finally capsule formation; eventually the worms were killed, eliminated, and the nodule disappeared.

In the skin of the palms and soles the adult female worm burrowed in the epidermis, producing an elongated serpiginous blood blister that eventually became purulent. In this blister the worm laid her eggs; and by the bursting of the blister the eggs were discharged into the outer world and placed in a position to infect new hosts. The reaction in the epidermis was evidently not severe enough to interfere seriously with the health of the host or with the continuation of the egg-bearing period of the female parasite. This condition of almost perfect parasitism is an ideal one for the continuation of the life of this species of nematode. In so far as we are able to determine this is the first description of a nematode that lays its eggs in the epidermis.

The provisional name of the parasite is *Trichosoma cutaneum*, 1922.

[2] In seeking to identify this parasite in literature we have consulted the Stiles and Hassall nematode catalogue (Stiles, C. W., and Hassall, A., *Bull. Hyg. Lab., U.S.P.H., No. 114, 1920*), Stossich (Stossich, M., *Boll. Soc. adriat. sc. nat. Trieste*, 1890, xii, 3), Travassos (Travassos, L., *Mem. Inst. Oswaldo Cruz*, 1915, vii, 146), and various other publications, but have found no record of cutaneous *Capillaria* in monkeys; neither has a record of this kind been found in the host catalogues of parasites in the Zoological Division of the United States Public Health Service and of the Bureau of Animal Industry.

For valuable assistance in this portion of the work and many helpful suggestions we are indebted to Professor Charles Wardell Stiles of the United States Public Health Service.

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## EXPLANATION OF PLATES.

### PLATE 39.

FIG. 1. Showing large and small subcutaneous nodules in the dorsum of the hand.

FIG. 2. Showing an elongated papular area above the left elbow.

FIG. 3. Showing a broad papule over the tendon of the triceps.

### PLATE 40.

FIGS. 4 and 5. Showing simultaneous swelling of the right ankle and left foot.

### PLATE 41.

FIGS. 6 and 7. Lateral view of the ankle and foot shown in Figs. 4 and 5.

### PLATE 42.

FIG. 8. Showing pitted edema of the dorsum of the left hand.

FIG. 9. Showing a very early small blister.

FIG. 10. Showing an elongated blister at the base of the great toe.

### PLATE 43.

FIG. 11. Showing ragged edged desquamating areas over the plantar region.

### PLATE 44.

FIG. 12. Early subcutaneous lesion showing a slight collar of cells about the worm and marked infiltration of eosinophils in the surrounding edematous tissue. × 230.

FIG. 13. Early subcutaneous lesion showing a slightly more marked collar of cells about the parasite and eosinophils in the neighboring edematous tissue. × 450.

PLATE 45.

FIG. 14. Early subcutaneous lesion showing the worm surrounded by fibrin and a few cells, in a tissue space, and perivascular reaction at some distance from the worm. × 55.

FIG. 15. Older subcutaneous nodule containing two cross-sections of a worm. *W*, worm (two cross-sections) surrounded by an intense cellular reaction; *PV*, perivascular reaction. × 85.

PLATE 46.

FIG. 16. Large globular subcutaneous nodule containing many sections of a worm, surrounded by a hemorrhagic zone. *HZ*, hemorrhagic zone; *CZ*, clear zone containing granular material and a few cells; *Y*, zone of young actively dividing cells; *P*, pedicle of the nodule containing many newly formed blood vessels; *PV*, perivascular reaction. × 85.

PLATE 47.

FIG. 17. Older subcutaneous nodule containing a single cross-section of a poorly staining worm. *W*, worm; *G*, giant cells surrounding worm; *C*, capsule; *PV*, perivascular reaction. × 85.

FIG. 18. Higher power of the portion of Fig. 17 showing the worm. *W*, worm; *G*, giant cells. × 450.

PLATE 48.

FIG. 19. Old subcutaneous nodule. × 85.

PLATE 49.

FIG. 20. Low power section of an early unbroken plantar blister containing a female nematode. *W*, worm; *WI*, cross-section of worm with very little necrosis of cells; *V*, microscopic vesicle containing only cell detritus. × 48.

PLATE 50.

FIG. 21. Unstained specimen of female *Trichosoma cutaneum* obtained from a palmar blister. *H*, head; *P*, posterior portion of body; *V*, vulva. × about 9.5.

FIG. 22. Head and esophagus. *H*, head; *E*, esophagus surrounded by cuboidal cells; *V*, vulva, from which eggs containing embryos are being expelled. × 37.

FIG. 23. Posterior end of the body containing the ovary and young ova. × 37.

PLATE 51.

FIG. 24. Eggs of *Trichosoma cutaneum*, each containing a well developed embryo. × 400.

FIGS. 25 and 26. Cross-section of a female *Trichosoma cutaneum* from a palmar blister. *M*, muscle cells; *N*, nerve cells; *I*, intestine; *O*, ovary; *U*, uterus containing partially matured eggs; *RS*, receptaculum seminis; *E*, empty egg-shell. × 410.

FIG. 27. Longitudinal section of a female parasite from a palmar blister. *RSO*, union of receptaculum seminis and ovary; *RS*, receptaculum seminis; O, ovary. × 400.

PLATE 52.

FIG. 28. Young worm in a subcutaneous nodule. *E*, esophageal cells, longitudinal section; *F*, cross-section of esophageal cells; *C*, cross-section of the posterior portion of the body. ×450.

FIGS. 29 to 31. Male (?) form of nematode in a subcutaneous nodule.

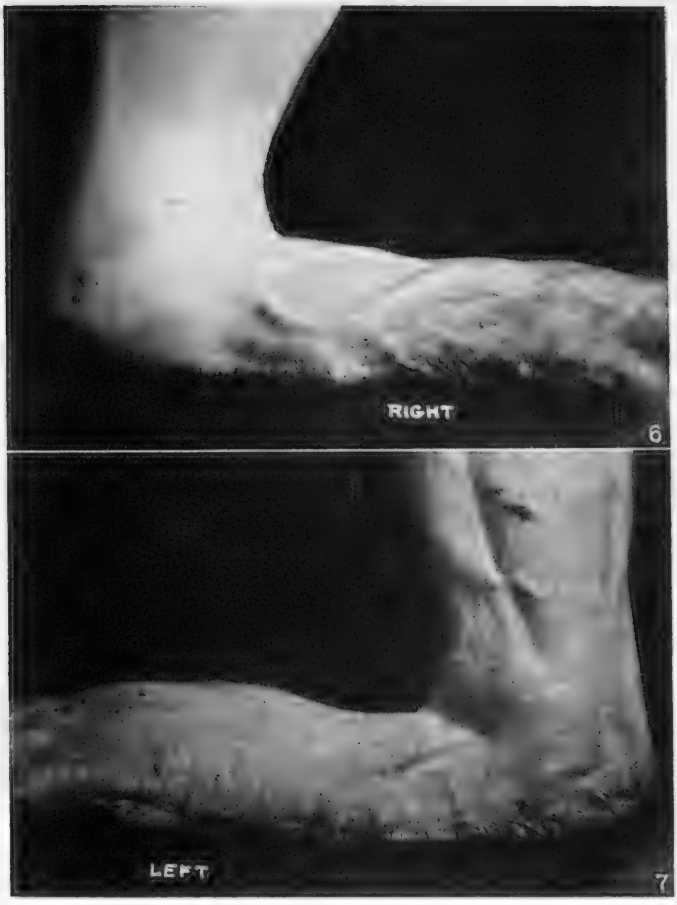
FIG. 29. Esophageal cells (*E*), longitudinal section. × 450.

FIG. 30. *E*, esophageal cell, cross-section; *I*, intestine; *G*, genital tract containing lumen. × 450.

FIG. 31. *M*, muscle cells; *N*, nerve cells; *I*, intestine; *G*, genital tract (testis?). × 450.

(Swift, Boots, and Miller: Cutaneous nematode infection in monkeys.)

(Swift, Boots, and Miller: Cutaneous nematode infection in monkeys.)

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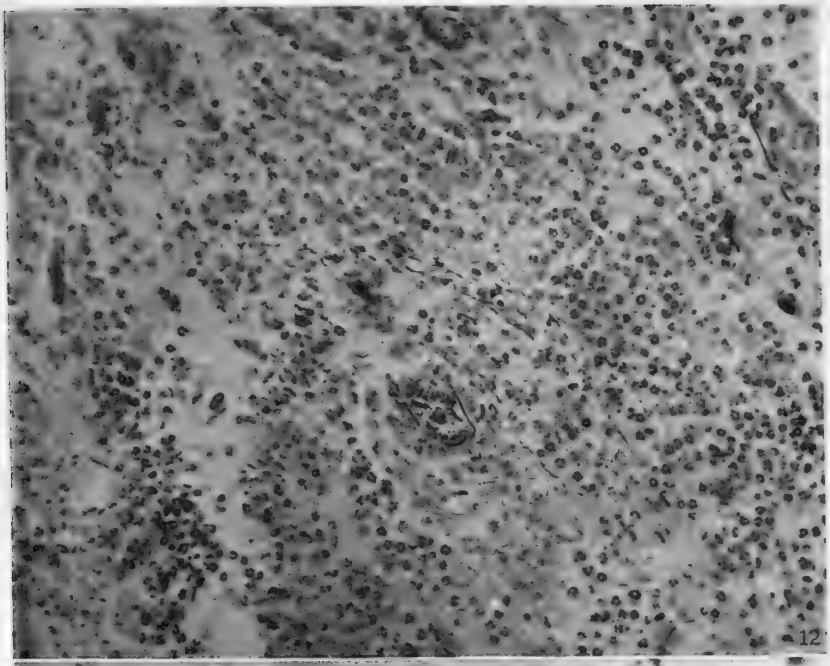

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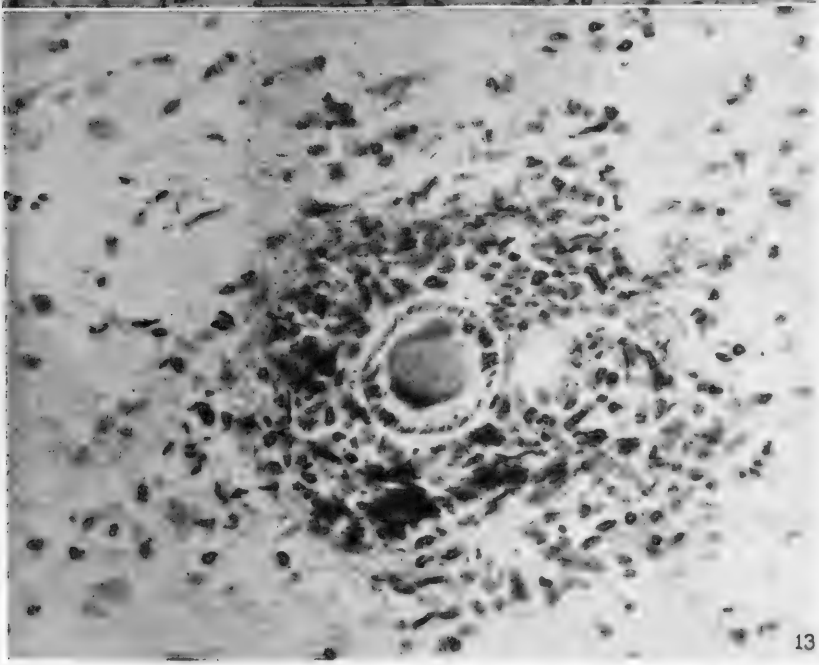

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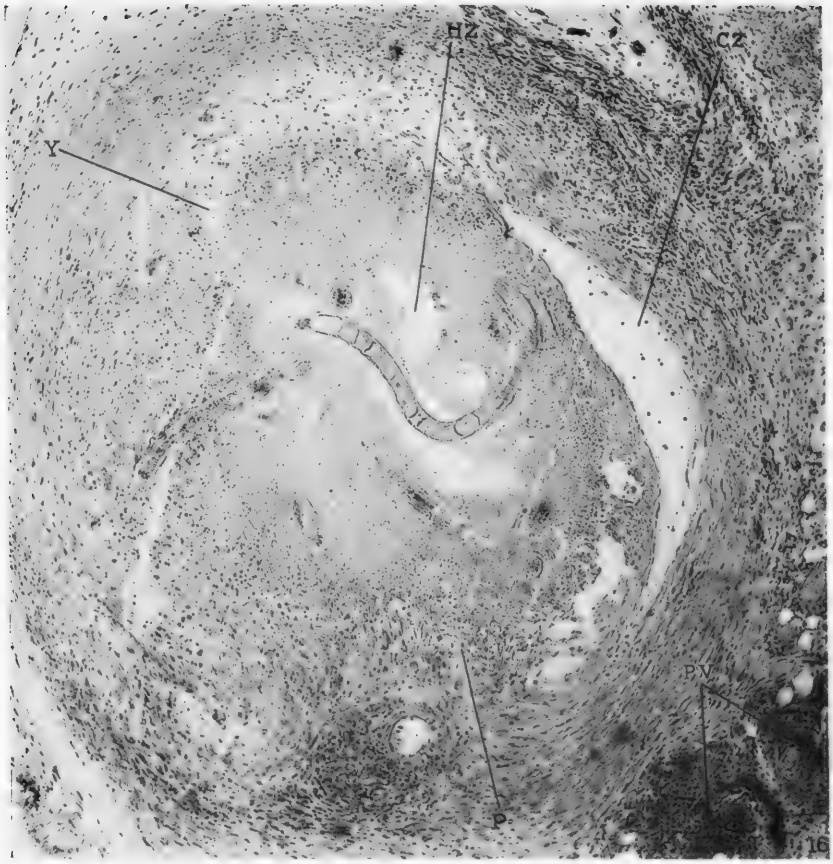
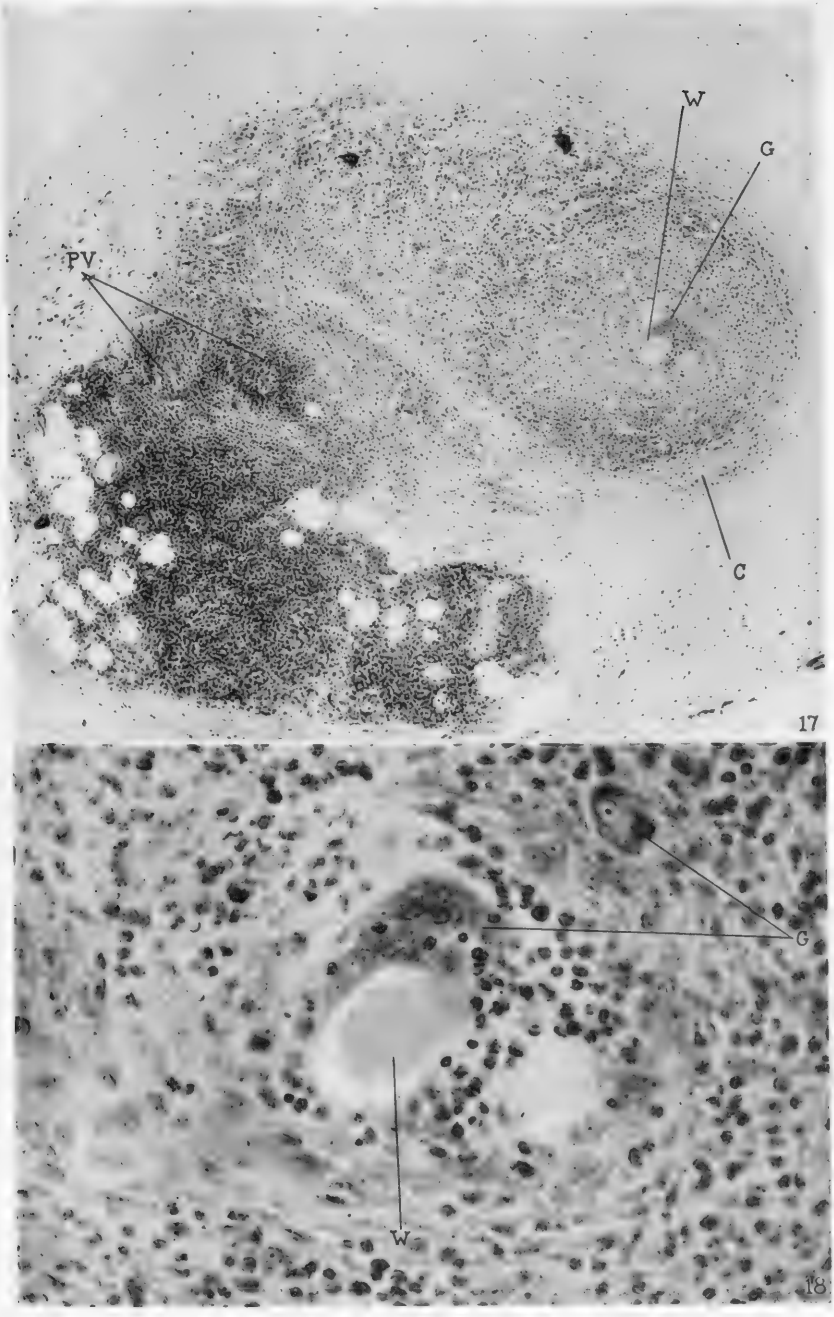



(Swift, Boots, and Miller, Cutaneous nematode infection in monkeys.)

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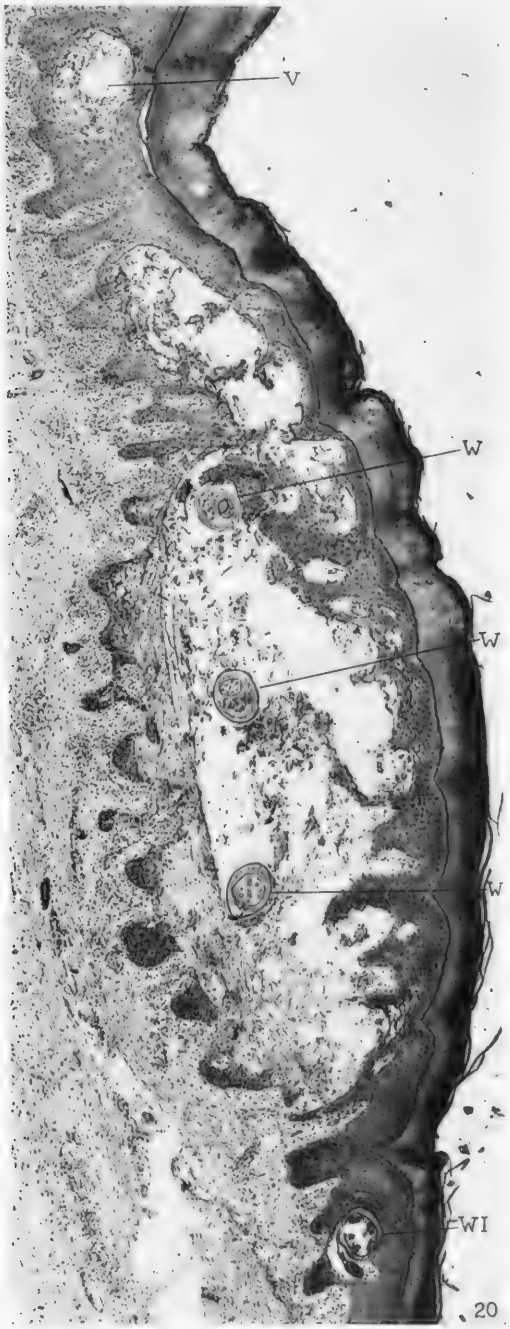
(Swift, Boots, and Miller: Cutaneous nematode infection in monkeys.)

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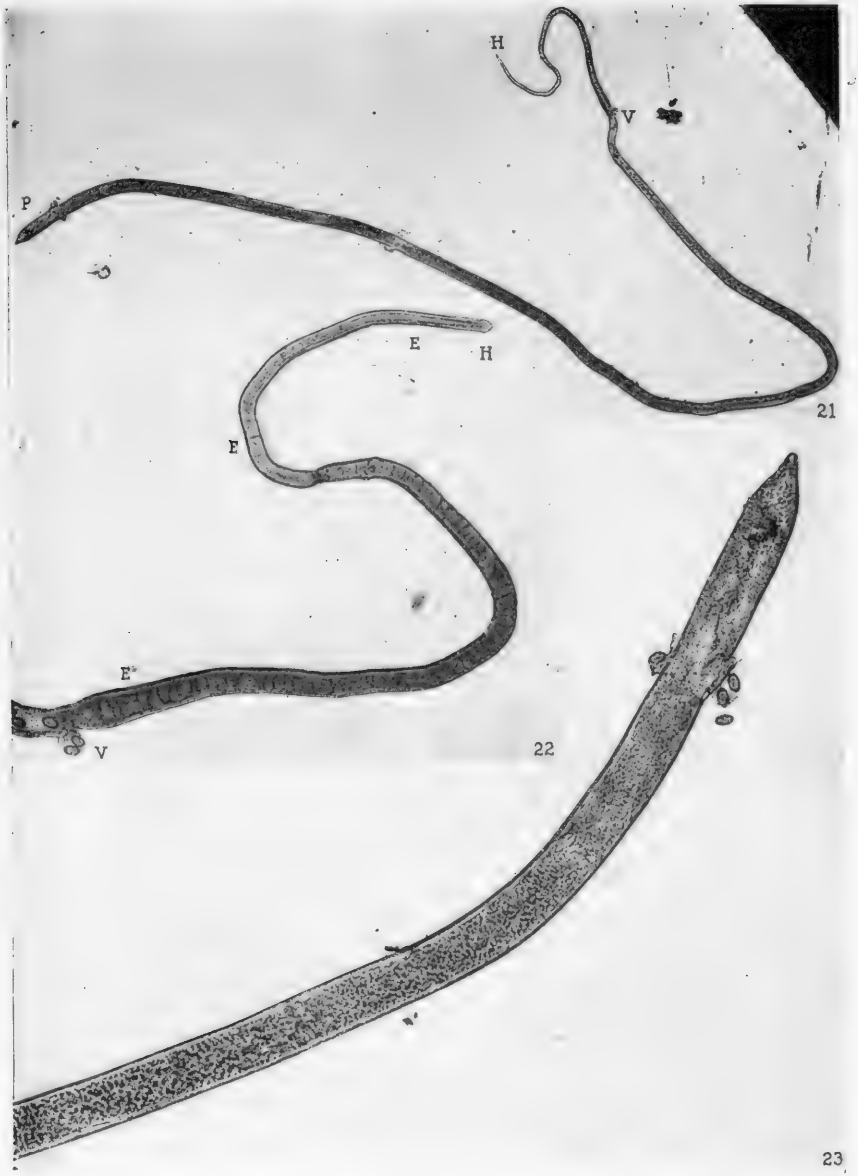


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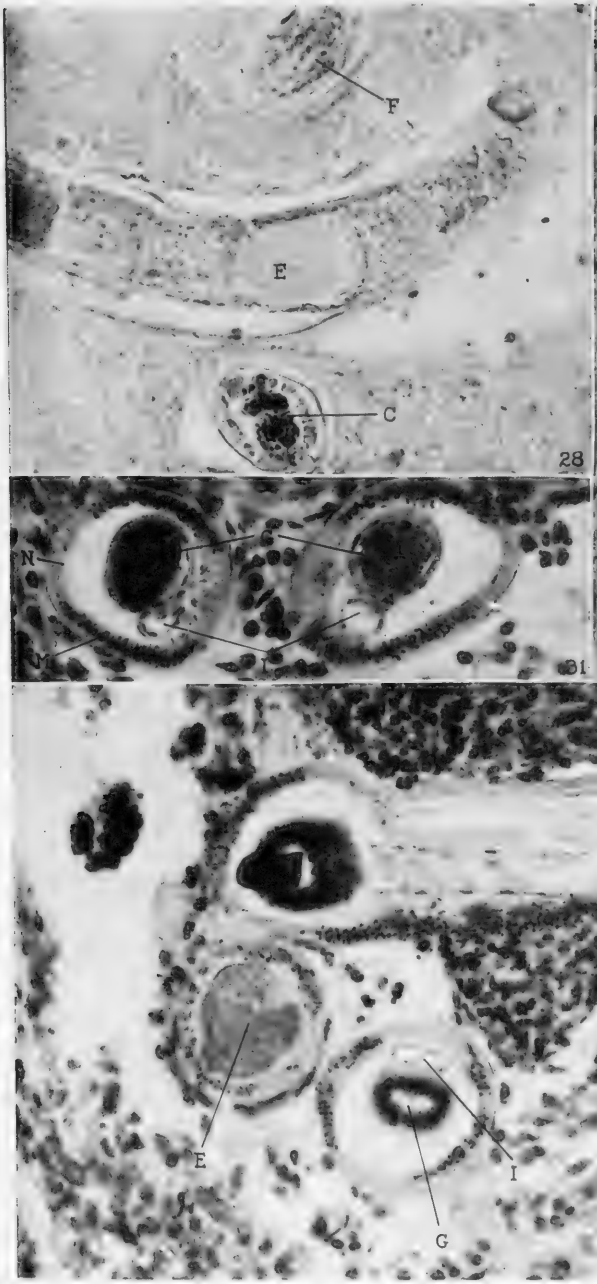


(Swift, Boots, and Miller: Cutaneous nematode infection in monkeys.)

# VIRULENCE AND MUTATION OF THE BACILLUS OF RABBIT SEPTICEMIA.

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## INTRODUCTION.

Types D and G, bacillus of rabbit septicemia, have been shown to possess greatly differing degrees of virulence (1). Type G, of low virulence, has been demonstrated to arise as a mutant from the primordial highly virulent Type D (2). It is the purpose of this paper to inquire into the fixity of the character of virulence of the two types and to determine the relation that mutation bears to this function of invasibility.

### *Method.*

The method of virulence determination employed in the following experiments is identical with that described in a previous paper (1). Briefly, it consists in the injection of varying amounts of 6 hour 10 per cent rabbit serum broth cultures, intrapleurally, into young rabbits of about 600 gm. weight. The dilutions of the test culture are carried out in plain broth, pH 7.4, and are increased by 10's. The dilutions are designated as follows: 0.0001 cc. of the original culture is written $10^{-4}$ cc., which of course is an abbreviation for $1 \times 10^{-4}$. The injections were invariably made immediately after dilution. The highest dilution was injected first, this procedure permitting the same syringe to be used for all injections. The final volume of each inoculation was in all cases 0.5 cc.

## EXPERIMENTAL.

The persistence of the character of virulence of Type D is demonstrated by the following observations.

*Experiment 1. Persistence of the Virulence of Strain R19, Type D.*—Strain R19, Type D, was isolated on October 20, 1920, from the heart's blood of a rabbit dead of bronchopneumonia. The necropsy showed the typical bronchopneumonia and fibrinopurulent pleuritis and pericarditis, caused by the rabbit septicemia bacillus. This culture was placed in 10 per cent rabbit serum agar and transplanted at intervals of 7 days—incubation at 37°C. for 8 hours, the remainder of the time in the ice box. Virulence was tested on November 5, 10, and 14, 1920, and February 2, 1921; that is, 16, 21, 25, and 105 days after isolation. The results are summarized in Table I.

TABLE I.

*Persistence of the Virulence of Strain R19, Type D.*

| Strain. | Length of time after isolation. | Weight of rabbit. | Age of serum broth culture injected. | Amount injected intrapleurally. | Result. |
|---|---|---|---|---|---|
| | *days* | *gm.* | *hrs.* | *cc.* | |
| R19-D | 16 | 550 | 6 | $10^{-5}$ | Died in 15 hrs.* |
| | | 550 | | $10^{-8}$ | " " 13 " |
| | | 560 | | $10^{-1}$ | " " 13 " |
| R19-D | 21 | 600 | 6 | $10^{-6}$ | " " 14 " |
| | | 650 | | $10^{-5}$ | " " 14 " |
| R19-D | 25 | 525 | 6 | $10^{-9}$ | Survived. |
| | | 515 | | $10^{-8}$ | Died in 12 hrs. |
| R19-D | 105 | 650 | 6 | $10^{-8}$ | " " 15 " |
| | | 650 | | $10^{-7}$ | " " 15 " |
| | | 650 | | $10^{-6}$ | " " 15 " |
| | | 650 | | $10^{-5}$ | " " 15 " |

* The necropsy showed typical bronchopneumonia, fibrinopurulent pleuritis, and pericarditis.

Table I indicates that the virulence of this strain remains at a very high level over a comparatively long period of time. The high dilutions ($10^{-8}$, $10^{-7}$) were plated in serum agar. $10^{-8}$ cc. yielded from three to eight colonies, showing the number of viable organisms in a 6 hour culture to vary from 300 to 800 million per cc. Strain R19-D was the most violently invasive of any of the cultures isolated. It is interesting to observe that the time of death of the test animals shows very little variation (12 to 15 hours). For the majority of virulent

Type D strains this is somewhat longer; *i.e.*, from 30 to 48 or 72 hours. Other Type D strains have been subjected to similar tests and, like Strain R19-D, exhibit a like constancy in maintenance of a high degree of virulence.

It was of interest to determine whether or not individual members of a single Type D culture were possessed of greatly varying degrees of virulence.

*Experiment 2. Virulence of Six Pure-Line Strains, Type D, Fished from a Single Culture.*—Six pure-line strains of Type D, R15, were fished by Barber pipette (3) from a 3 hour undiluted rabbit serum culture. These were seeded into undiluted rabbit serum and after several passages in this medium were transplanted to 10 per cent rabbit serum broth, incubated for 6 hours at 37°C., and tested for virulence by the usual technique. The results are summarized in Table II.

TABLE II.

*Virulence of Six Pure-Line Strains, Type D, Fished from a Single Culture.*

| Strain. | Weight of rabbit. | Age of serum broth culture injected. | Amount injected intrapleurally. | Result. |
|---|---|---|---|---|
| | *gm.* | *hrs.* | *cc.* | |
| B-$D_1$ | 600 | 6 | $10^{-6}$ | Died in 36 hrs.* |
| | 580 | | $10^{-4}$ | " " 36 " |
| B-$D_2$ | 600 | 6 | $10^{-6}$ | " " 36 " |
| | 600 | | $10^{-4}$ | Survived. |
| B-$D_3$ | 610 | 6 | $10^{-6}$ | Died in 48 hrs. |
| | 590 | | $10^{-4}$ | " " 47 " |
| B-$D_4$ | 600 | 6 | $10^{-6}$ | " " 36 " |
| | 600 | | $10^{-4}$ | " " 36 " |
| B-$D_6$ | 610 | 6 | $10^{-6}$ | " " 36 " |
| | 610 | | $10^{-4}$ | " " 48 " |
| B-$D_8$ | 600 | 6 | $10^{-6}$ | " " 36 " |
| | 590 | | $10^{-4}$ | " " 36 " |

* The necropsies were typical in all cases.

Table II indicates that there is very little variation in different individuals of a Type D culture. All of the six serum broth subcul-

tures from the pure-line strains were streaked on serum agar at the time of the virulence test and were found to contain Type D organisms only.

It is well known that various microorganisms attenuate quickly when they are isolated from the animal body and grow upon artificial media. This loss of virulence appears especially rapidly when the media employed are not enriched with blood, serum, or ascitic fluid. Wadsworth and Kirkbride (4) found that the virulence of pneumococci could be maintained at a high level for a long period when the culture was transplanted continuously at 8 hour intervals in plain broth, but that it quickly decreased when 24 hours were allowed to elapse between passages. Various observations having indicated that the virulence of Type D shows little variation, an experiment was planned to determine whether decidedly unfavorable conditions would depress this function. It has been shown in a previous communication that pure-line strains of Microbe D undergo mutation into Type G when allowed to stand without transplant for some days in plain broth. This process does not, however, go to completion under these conditions. A certain proportion of Type D remains as is evidenced by streaking such cultures on serum agar plates. It was proposed, therefore, to test the virulence of those Type D organisms which remain without change and at the same time to find out whether the newly mutated Type G colonies would yield cultures of characteristically low invasive power.

*Experiment 3. Persistence of the Virulence of Type D in Broth Cultures Kept for 9½ Days at 37°C. without Transplant; Lack of Virulence of Type G Mutants Arising in This Culture.*—0.05 cc. of an 18 hour rabbit serum culture, Strain A-D, R15, was seeded into a large test-tube containing 10 cc. of plain broth, pH 7.4. The tube was capped with tin-foil and placed in the incubator at 37°. The tube was removed from the incubator at 24 hour intervals and a loop of its contents streaked on 10 per cent rabbit serum agar plates by the method described in a preceding paper (2). Type G colonies began to appear on the plates of the 52 hour test, and at 228 hours (9½ days) Type G showed preponderance over Type D, the ratio between D and G being 40:60. Characteristic fluorescent Type D and non-fluorescent Type G colonies were now carefully fished to 10 per cent rabbit serum broth. Good growth had occurred in broth of the subcultures at 8 hours, the appearance of Type D being diffusely turbid, that of Type G very granular. Both tubes were now carefully shaken up, diluted to $10^{-7}$ cc. of the

original in plain broth, pH 7.4, and immediately injected intrapleurally into 700 gm. rabbits. The results of this experiment are recorded in Table III.

TABLE III.

*Persistence of the Virulence of Type D in Broth Cultures Kept for 9½ Days at 37°C. without Transplant; Lack of Virulence of Type G Mutants Arising in This Culture.*

| Type. | Age of serum broth culture injected. | Weight of rabbit. | Amount injected intrapleurally. | Result. |
|---|---|---|---|---|
| | *hrs.* | *gm.* | *cc.* | |
| Type D; from plain broth culture; 228 hrs.; 37°C. | 8 | 700 | $10^{-7}$ | Died in 42 hrs.* |
| | | 710 | $10^{-6}$ | " " 36 " * |
| | | 700 | $10^{-5}$ | " " 36 " * |
| | | 690 | $10^{-4}$ | " " 36 " * |
| | | 700 | $10^{-3}$ | " " 42 " * |
| | | 720 | $10^{-2}$ | " " 22 " * |
| | | 680 | $10^{-1}$ | " " 18 " * |
| Type G; from plain broth culture; 228 hrs.; 37°C. | 8 | 700 | $10^{-4}$ | Survived. |
| | | 710 | $10^{-3}$ | " |
| | | 700 | $10^{-2}$ | " |
| | | 710 | $10^{-1}$ | " |
| | | 700 | $5 \times 10^{-1}$ | Died in 15 days. † |

* Necropsy typical.

† Necropsy showed no evidence of infection; heart's blood sterile.

It will be observed from Table III that the subculture from the Type D colony was very virulent. Injected over a range of dilutions from $10^{-7}$ to $10^{-1}$ cc., it proved fatal in every case. On the other hand, 0.1 cc. of the subculture from the Type G colony failed to produce an appreciable ill effect. The rabbit receiving 0.5 cc. succumbed in 15 days, but necropsy failed to reveal the characteristic bronchopneumonia and fibrinopurulent pleuritis and pericarditis, and the heart's blood culture proved sterile. This experiment is a clear demonstration that Microbe D is able to preserve its virulence under conditions generally considered to be unfavorable, while the mutant Type G forms that have arisen in the same culture show the lack of virulence that characterizes this type. It is apparent from the foregoing experiment that the virulence of a culture containing both Types D and G is proportional to the number of the D variety remaining in the culture.

If this be true, it is conceivable that the attenuation of a Type D culture in plain broth may be due to a D → G mutation and gradual increase in the number of Type G individuals. This augmentation might take place either by a constantly increasing tendency of the Type D forms to mutate, or by an actual overgrowth of Type D by the mutant G variety. If Type G should come finally to supplant the parent Type D completely, the formerly virulent culture should be almost completely attenuated. This idea was put to test in Experiment 4.

*Experiment 4. Relation of the Virulence of a Plain Broth Culture to the Proportions of Types D and G Present.*—Microbe D,R15, was transplanted daily for 25 days in plain broth, pH 7.4. From time to time the culture was streaked on 10 per cent serum agar plates. No Type G forms were observed. At the twenty-fifth passage the virulence of the culture was tested by the usual technique. At the thirtieth plain broth passage a few Type G colonies were detected on the serum agar plate. The culture was now transplanted every 48 hours. The relative number of Type G colonies increased rapidly. At the 51st passage Type G was found to preponderate greatly over Type D. The virulence of the whole culture was again tested at this point. The period of time between transplants was lengthened to 5 days. At the 56th passage serum agar plates revealed nothing but Type G. Microbe D had completely disappeared. The virulence was again tested. The results of the experiment are summarized in Table IV.

The results of Experiment 4, as seen in Table IV, can be summed up briefly as follows: At the twenty-fifth passage in plain broth no Type G colonies had arisen and the virulence was approximately equal to that of the original serum broth culture of Type D, $10^{-6}$ cc. proving fatal to 600 gm. rabbits. At the 51st passage, 2 months later, there was a large preponderance of Type G. The virulence had fallen to $10^{-4}$ cc. At the 56th passage, 5 days later, no Type D colonies could be demonstrated. Either complete D → G mutation had occurred, or the originally mutated Type G had completely outgrown Type D. 0.1 cc. of the culture failed to produce a fatal effect. The attenuation of this culture is to be referred to the gradual replacement of the primordial Type D by the mutant G form. It is possible to predict the virulence of a culture from the proportion of the two types present, as evidenced by the serum agar plate streak method. It is possible to procure subcultures of very high or very low virulence by selection of one type or the other, so long as any of Type D remain.

While the virulence of Type G, isolated from Type D cultures, is very low, large amounts of undiluted culture may occasionally cause fatal infections, especially in young rabbits. Adult rabbits of 1,200 to 1,800 gm. weight have not been observed to succumb, even when

TABLE IV.

*Relation of the Virulence of a Plain Broth Culture to the Proportions of Types D and G Present.*

| No. of passages. | Interval between transplants. | Proportion of Types D and G. | Amount injected intrapleurally.* | Result. | Necropsy. |
|---|---|---|---|---|---|
| | *days* | | *cc.* | | |
| 25 | 1 | D only. | $10^{-6}$ | Died in 36 hrs. | Typical. Type D in heart's blood. |
| | | | $10^{-5}$ | " " 24 " | Typical. Type D in heart's blood. |
| | | | $10^{-4}$ | Survived. | |
| | | | $10^{-3}$ | Died in 31 hrs. | Typical. Type D in heart's blood. |
| 51 | 2 (All passages from 25th to 51st at 2 day intervals.) | D + G; G greatly preponderates. | $10^{-6}$ | Survived. | |
| | | | $10^{-5}$ | " | |
| | | | $10^{-4}$ | Died in 36 hrs. | Typical. Type D in heart's blood. |
| | | | $10^{-3}$ | Survived. | |
| | | | $10^{-2}$ | Died in 36 hrs. | Typical. Type D in heart's blood. |
| | | | $10^{-1}$ | " " 36 " | Typical. Type D and few Type G in heart's blood. |
| 56 | 5 (All passages from 51st to 56th at 5 day intervals.) | G only. | $10^{-5}$ | Survived. | |
| | | | $10^{-4}$ | " | |
| | | | $10^{-3}$ | " | |
| | | | $10^{-2}$ | " | |
| | | | $10^{-1}$ | " | |

* All rabbits 600 to 650 gm. in weight. Cultures 6 hour, plain broth.

1.0 cc. of a whole culture of Type G has been injected. On the other hand, rabbits of 600 gm. weight at times are fatally infected by 0.5 cc., more often by 1.0 cc. of undiluted serum broth culture.

*Experiment 5. Increase of the Virulence of Type G by Passage through Rabbits.*—Microbe G was isolated from Strain R15-D and carried for thirty-eight passages in serum broth. It was then tested in the usual manner for virulence on rabbits of 600 gm. weight. Three rabbits of this series, injected with large doses, succumbed to typical infections indistinguishable from those caused by Type D. Pure Type G cultures were isolated in all cases. One of these was carried for five passages in serum broth and then retested for virulence. A strain of Type G, isolated from rabbits succumbing to the second virulence test, was carried in serum broth for fifteen additional passages, daily transplant, and again subjected to virulence test. The results are summarized in Table V.

The results summarized in Table V indicate that the virulence of Microbe G can be raised by passage. While the virulence undoubtedly increases, the other characteristics differentiating Type G from Type D persist and even intensify. This is especially true of the granular growth character in fluid medium. This becomes very intense upon animal passage, the organisms of serum broth cultures clumping into solid masses that fail to break up even after prolonged shaking of the culture tube. The acid agglutination zone also broadens considerably. Thus in the case of one of these intensely granular strains, a four times washed suspension of Microbe G agglutinated completely in all the tubes of an acetic acid-sodium acetate buffer series, from pH 5.6 to 3.0.

The experiment demonstrates that granular growth character and lack of virulence do not necessarily always occur concomitantly. It had been supposed that there might be some causal connection between the instability of the organisms and their lack of virulence, but this idea would seem to be ruled out by Experiment 5.

It will be observed that in the case of the last animal of the series in Table V some Type D colonies were observed on the serum plates streaked from the pleural fluid at necropsy. Do these represent reversion of Type G to the primordial D form? It is impossible to say whether or not this is the case. This is true because rabbits in many cases harbor Type D normally. The Type D discovered at necropsy might, therefore, simply represent individuals which have invaded the pleural cavity when the animal is already nearly dead from a true Type G infection. It is this tendency of normal rabbits to carry Microbe D that makes it unfeasible to attempt the reversion of Type G to the parent D variety. It is certainly true that Type G

TABLE V.

*Increase of the Virulence of Type G by Passage through Rabbits.*

| Strain. | No. of passages. | Weight of rabbit. | Amount injected intrapleurally. | Result. | Necropsy. |
|---|---|---|---|---|---|
| | | *gm.* | *cc.* | | |
| R15-G | 0 | 600 | 0.1 | Survived. | |
| | | 610 | 0.5 | Died in 24 hrs. | No necropsy. |
| | | 600 | 1.0 | Survived. | |
| | | 600 | 2.0 | Died in 32 hrs. | Typical fibrinopurulent pleuritis, pericarditis, and bronchopneumonia. |
| | | 600 | 3.0 | " " 5 days. | Pure culture Type G recovered from heart's blood. |
| R15-G | 1 | 620 | $10^{-3}$ | " " 16 hrs. | Congested lungs; increase in pleural fluid. Type G found in heart's blood at necropsy. |
| | | 610 | $10^{-2}$ | " " 16 " | Congested lungs; increase in pleural fluid. Type G found in heart's blood at necropsy. |
| | | 610 | $10^{-1}$ | " " 19 " | Beginning bronchopneumonia and pleuritis. Type G found in heart's blood at necropsy. |
| | | 600 | $5\times10^{-1}$ | " " 16 " | Congested lungs; increase in pleural fluid. Type G found in heart's blood at necropsy. |
| R15-G | 2 | 700 | $10^{-6}$ | Survived. | |
| | | 750 | $10^{-4}$ | Died in 24 hrs. | Lungs congested; pleural fluid increased. Heart's blood pure Type G. |
| | | 725 | $10^{-2}$ | " " 36 " | |
| | | 700 | $10^{-1}$ | " " 15 " | Lungs congested. Type G + *few Type D* found in heart's blood. |

is by itself able to cause fatal infections when injected in overwhelming dose. This was the case in all of the other animals whose necropsies are recorded in the table.

What is the cause of the loss of the property of invasibility in the case of Type G? It was considered possible that Types D and G

might differ in degree of phagocytability or in their aggressive action against white blood cells. This idea was put to test in Experiment 6.

*Experiment 6. Intrapleural Reaction to the Injection of Large Amounts of Types D and G.*—9 hour serum broth cultures of Microbes D and G were carefully shaken up. 1 cc. of each of these was added to 1.5 cc. of plain broth. The suspensions of Type D and of Type G (total volume 2.5 cc.) respectively were injected intrapleurally into each of two rabbits of 1,600 gm. weight. Pleural fluid was aspirated after 1, 2, and 4 hours, and smears were made of the withdrawn fluid and stained with Löffler's methylene blue. The results of this experiment are given in Table VI.

TABLE VI.

*Intrapleural Reaction to the Injection of Large Amounts of Types D and G.*

| Interval before examination. | Microbe D. | Microbe G. |
|---|---|---|
| | 9 hr. serum broth culture intrapleurally in 1,700 gm. rabbit. | 9 hr. serum broth culture intrapleurally in 1,700 gm. rabbit. |
| *hrs.* | | |
| 1 | No fluid obtainable. | No fluid obtainable. |
| 2 | Large amount of fluid; myriads of typical bipolar organisms; no slight phagocytes. | Small amount of fluid; no phagocytes; a few clumps of typical bipolar organisms. |
| 4 | Myriads of typical organisms; few phagocytes, many badly damaged; slight phagocytosis. | Many phagocytes with ingested organisms; no free organisms observed. |
| 16 | Dead; myriads of organisms; some phagocytosis. | No free organisms; few phagocytes. Survived. |

It would appear from the experiment just described that Type D possesses a strong antagonistic effect upon the phagocytes, which allows the microbes to gain their primary foothold in the pleural cavity and undergo intense multiplication. The mutant Type G organisms appear to have lost this aggressive activity and are rapidly clumped and ingested by the white blood cells, which appear at the injection site within 4 hours after inoculation. These facts would seem to furnish a clue for the study of possible aggressive substances possessed by Microbe D.

It has just been shown that Type G is rapidly disposed of when injected intrapleurally. It was interesting to observe that a culture of Type G which produced no perceptible effect when injected into

rabbits intrapleurally in dose of 1.0 cc., gave rise to abscesses when injected subcutaneously in 0.1 cc. amount. These abscesses were sharply circumscribed, purulent, and teeming with typical G microbes. In no case did the organisms generalize and death result. Type G could be recovered from them for several weeks. This demonstrates a vicariously greater susceptibility to infection depending upon the route of injection. It is well known that phagocytes are more rapidly mobilized on serous surfaces than subcutaneously, and in the light of Experiment 6, the phenomenon of greater infectivity of Type G by subcutaneous route would seem to be due to greater time necessary to bring the phagocytes into play in this region.

## DISCUSSION.

The importance of the foregoing observations and experiments is threefold.

1. Various authors have observed the appearance of granular growing varieties in cultures of various microorganisms. Such types have been reported by Arkwright (5) and by Zoeller (6) for *Bacillus dysenteriæ* Shiga. They have been noted in the case of the hog-cholera bacillus by Krumwiede and Provost[1] and by Krumwiede and Valentine for pneumococcus.[2] This phenomenon is not therefore confined to cultures of the rabbit septicemia bacillus and may be found to be of general occurrence. It would be of interest to observe whether lack of virulence is associated with granular growth in these instances.

2. The occurrence, side by side, of the two varieties, the parent Type D, highly virulent, and its mutant Type G, of low invasibility, furnishes an excellent opportunity for the study of the mechanism of virulence. It is possible to investigate the products of secretion and of cell destruction of the virulent Type D, with a view to discovering whether these substances, when added to Type G, might increase its power of invasion. In brief, Type G may be used as a reagent with which to attempt the discovery of the factors responsible for the high invasive power of Type D.

[1] Personal communication.
[2] Personal communication.

3. It is important to find out whether the G forms appear only in strains which have been artificially cultured for some time outside the animal body, or whether they may occur naturally in the animal as well as in the test-tube. This question would seem to have a direct bearing upon the problem of epidemiology. It is at present under investigation and certain results have already been obtained, which will be reported in the near future.

## SUMMARY AND CONCLUSIONS.

Type D, bacillus of rabbit septicemia, exhibits marked fixity of the character of virulence. This is true for cultures that are regularly transplanted on serum agar or in broth. It is also to be observed when organisms of this variety are subjected to unfavorable conditions; *e.g.*, remaining for $9\frac{1}{2}$ days without transplant in plain broth. Under such conditions no decrease in virulence was observed. G forms which arose in the same culture during this time exhibited characteristic lack of invasibility.

Different individuals of a given culture of Type D do not vary to a noticeable extent in virulence. This was ascertained by test of virulence of cultures arising from six individuals fished from Type D cultures by Barber pipette.

A Type D culture subjected to passage in plain broth undergoes D → G mutation. Type D and G individuals can be demonstrated to be present in the same culture. The virulence of such a mixed culture is proportional to the relative member of Microbes D and G present. This throws light on certain mechanisms of attenuation.

The virulence of Type G can be raised to a considerable titer by animal passage. Such organisms do not, however, lose their characteristic of granular growth. This last, on the other hand, appears to intensify by animal passage. The acid agglutination zone of Type G strains which have been passed through animals shows a marked broadening.

Microbe D owes its superior invasive power at least in part to its antiphagocytic activity, a property apparently not possessed by Microbe G.

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# COLLODION SACS FOR AEROBIC AND ANAEROBIC BACTERIAL CULTIVATION.

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PLATES 53 AND 54.

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Among the various media for bacterial cultivation, one that has proved especially valuable for the isolation and maintenance of highly parasitic organisms is a combination of ascitic fluid or dilute blood serum and a fragment of fresh animal tissue. Such a medium was suggested by Theobald Smith[1] and has been extensively developed and used by Noguchi[2] in the cultivation of spirochetes, the globoid bodies of poliomyelitis, and other organisms whose isolation in pure culture he has reported from time to time. *Bacterium pneumosintes*,[3] recovered by Olitsky in 1918 from the filtered nasopharyngeal secretions of patients with epidemic influenza, was isolated under strictly anaerobic conditions in a combination of sterile human ascitic fluid and fresh rabbit kidney tissue.

A certain disadvantage of the medium is found in the presence of protein precipitate derived from autolysis of the tissue fragment. Both macroscopically and in stained specimens this precipitate may obscure the view and either simulate or mask the presence of significant bodies. This disadvantage is apparent especially in the search for hitherto undiscovered microbes and in dealing with filter-passing organisms such as the globoid bodies of poliomyelitis or *Bacterium pneumosintes*, which are just within the limits of vision and are made out with difficulty in early generations. Furthermore, the antigenic protein precipitate makes tissue cultures unsuitable for the production

[1] Smith, T., *J. Boston Soc. Med. Sc.*, 1898–99, iii, 340.

[2] Noguchi, H., *J. Exp. Med.*, 1911, xiv, 99.

[3] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 713.

of immune serum or for serological studies in general, because it gives rise to unspecific reactions and so complicates the results.

In the course of recent studies on *Bacterium pneumosintes*, it became desirable to cultivate the organism in a medium without antigenic properties and free from the confusing protein cloud. For this purpose it was proposed to confine the tissue medium in a collodion sac surrounded by distilled water or salt solution, with the prospect that the nutritive and growth-promoting substances of the medium would diffuse through the sac wall and support growth in the surrounding fluid. The design of the apparatus was limited by the necessity for oxygen exclusion and for adjustments of osmotic pressure, so that the collodion sacs prepared for intraperitoneal implantation[4] were found to be unsuitable. It was also desirable that the dialysate cultures might be made in considerable numbers and be subject to frequent examination without disturbance of their anaerobic state. These conditions were met in the method to be described. The apparatus is adapted to various problems in bacterial growth and metabolism, and may therefore find a wider application than we originally intended. Two designs of apparatus have been developed, one containing about 10 cc. of culture medium for routine cultivation, the other holding 50 cc. for mass cultures. The principles involved are the same, but differences in preparation necessitate separate descriptions.

### *Collodion Sacs in V-Tubes.*

For small amounts of culture media a V-shaped glass tube[5] similar to the Smith fermentation tube, except that both ends are open, incloses the collodion sac and its surrounding fluid. The sac almost fills one limb of the V, reaching to the bend at the bottom, and is surrounded by the dialysate fluid, which rises a short distance in the other limb and is thus accessible from its open end. The mouth of the sac is shrunk onto a glass tube sealed into the V-tube by a rubber collar (Text-fig. 1). For anaerobic cultivation the medium in the sac and the dialysate fluid are layered with vaseline, or the V-tube may be placed in an anaerobic jar.

[4] Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 25.

[5] Kimble Glass Co., Vineland, N. J., or Corning Glass Works (Pyrex), Corning, N. Y.

TEXT-FIG. 1. Cross-section of a collodion sac in its V-tube ready for sterilization.

*Preparation of the Collodion Sacs.*

The preparation of a considerable number of identical collodion sacs in the simplest and most mechanical manner involves the assembly of some accessory apparatus which adds greatly to the ease and rapidity of the method.

The molds in which the sacs are formed are clusters of five short round bottom test-tubes 10 by 1.4 cm. inside, thrust to the lip through holes in a large (No. 13) rubber stopper (Fig. 1). This stopper fits the mouths of three cylindrical museum jars, 5.5 by 15 cm., provided with glass tops, gaskets, and clamps.[6] One jar contains a 10 per cent solution of gelatin, preserved with 0.3 per cent tricresol; the second contains a thick collodion solution; the third is partly filled with alcohol.

[6] Whitall Tatum Co., New York, N. Y., Fig. 2600, No. 2.

For the purpose of drying the gelatin coating and the collodion membrane with which the molds are lined, we have set up an apparatus to blow air into the molds. Short stubs of glass tubing are thrust through a No. 13 rubber stopper in a pentagonal pattern corresponding to the molds. This stopper is set in a small glass funnel for the delivery of air, and the funnel and stopper are supported in a ring clamp and held in place with adhesive plaster. A second ring clamp supports the rubber stopper containing the cluster of molds above the glass tips, so that a jet of air may be directed into each.

The molds are first lined with a thin coat of gelatin, which prevents the collodion membrane from adhering to the glass. The ease with which the completed sacs are withdrawn from the molds justifies this preliminary procedure. To line the molds, which must be clean and smooth, the gelatin solution is melted in a bath of warm water, the cluster of molds is fitted to the mouth of the jar, and the jar is momentarily inverted. The excess gelatin drains back into the jar and any bubbles in the gelatin break while the film is cooling. This thin coat of gelatin dries in a few minutes over the air jets described above. A number of mold clusters are prepared before the collodion sacs are made.

The collodion solution should be so thick and viscous that a membrane of sufficient thickness and strength will adhere to the mold even after 3 to 5 minutes drainage. Such a thick collodion is prepared most easily by evaporation of a thinner solution in a partial vacuum with gentle heat, as described in a former paper.[4]

To form the collodion membranes, a cluster of molds is fitted to the museum jar containing collodion, the jar is inverted momentarily, and the excess liquid is allowed to drain back into the jar until drops no longer form and fall. It may be necessary to hold the stopper tightly in place during the inversion, as evaporation of the solvents produces a slight pressure in the jar. A long drainage produces walls of more uniform thickness and allows even minute bubbles in the membranes to drain out or to break.

The collodion-coated molds are then removed from the jar, which is quickly covered again, and are held over the air jets for a few seconds until the ether vapor is washed out and the collodion sets. As was pointed out in the former paper,[4] the membranes must not be allowed

to dry completely if their permeability is to be retained after heat sterilization. As soon as the collodion sets, the molds are filled with alcohol by inversion of the third museum jar. Here they are allowed to soak for about 10 minutes, during which time the jar may be reversed two or three times to bring fresh alcohol into the tubes.

The molds are then rinsed out several times in cold tap water and immersed for a few minutes while the water penetrates the membranes and softens the gelatin beneath. During this immersion in cold water the lips of the tubes are scraped with a knife to cut the sacs free from the layer of collodion on the rubber stopper and each sac is fitted with the glass tube which is to support it. These glass tubes, 16 by 1.3 cm. outside, with an elastic band snapped on 2 cm. from one end, are of such a size in relation to the molds that they will just slip down into the sacs until checked by the elastic band.

When the gelatin lining under the collodion has softened, the molds with the glass tubes surmounting them are lowered for a minute or two into boiling water in a beaker or water bath. A wire hook (Fig. 1) which engages a small staple centrally placed among the lips of the molds is convenient for this purpose. The hot water shrinks the sacs down tightly upon the glass tubes, melts the gelatin, and so frees the sacs from the molds, so that when the tubes are withdrawn, the sacs come with them. The elastic bands are slipped down over the upper edge of the sacs, holding them in place securely.

The sacs are then rinsed in warm water to remove any traces of gelatin. When empty, they may be tested for leaks by inflation under water. They are now ready for insertion into the V-tubes. These tubes, of 1.5 cm. internal diameter, have limbs 6 and 12 cm. long. A wet collar of pure gum rubber tubing is slipped onto the shorter limb so that it does not obstruct the open end, into which the sac is thrust until its glass-protected neck is in the opening and its closed end has reached the curve at the bottom of the V-tube. The collar is then slipped up to hug the neck of the sac, which is allowed to project a millimeter or two above the collar. Thus the sac is held in place and the inner and outer chambers of the apparatus are sealed (Text-fig. 1). If rubber tubing of the proper size is not available, the collar may be made of a piece of thin rubber dam, 15 by 5 cm., wrapped tightly around the juncture of the V-tube and the sac, and secured with rubber bands.

The sac is then filled and surrounded with distilled water or saline solution, the ends of the tubes are plugged with cotton, and the completed apparatus is sterilized in the autoclave. Finally, the junction between rubber collar, sac, and tube is painted with hot paraffin wax. The V-tubes are carried in test-tube racks cut with slots instead of round holes.

*Collodion Sacs in Flasks.*

For mass cultures, collodion sacs of about 50 cc. capacity have been prepared. The molds are 50 cc. Erlenmeyer flasks, lined with a gelatin film. The melted gelatin is poured from one flask to another until all are coated. They are drained mouth downward in a rack (Fig. 2), until the gelatin cools and sets. The film is then dried over a jet of air. Collodion is kept in 120 cc. wide mouth rubber-stoppered bottles, into which the filled flasks are allowed to drain until all bubbles have broken and the excess collodion has dripped away. The subsequent treatment is the same as with the smaller sacs. Rather larger glass tubes, 16 by 1.6 cm., are required to support the sacs, and the elastic bands must be stretched tightly to prevent leaks between membrane and tube. The band is put on by collapsing the sac in a 50 cc. centrifuge tube or wide test-tube, around which the rubber band has been tightly doubled and wound. The neck of the sac, with the glass tube in position, projects from the centrifuge tube. The rubber band is then snapped off into place.

As containers, we have had glass tubes 12 by 1.5 cm. fused into 100 cc. Pyrex Erlenmeyer flasks near the bottom, thus forming a spout which gives access to the space surrounding the sac. The sac is collapsed, inserted into the flask, and sealed in place by an elastic collar of tubing or rubber dam. The necks of sacs made in ordinary 50 cc. flasks are not long enough to extend above this collar, hence the necessity for a tight rubber band around the neck. We have had no leakages which could be traced to this source. As with the smaller sacs, the apparatus is partly filled with distilled water or salt solution, plugged with cotton, and autoclaved. The juncture of collar and tube is then paraffined.

*Additional Notes on the Preparation and Use of the Sacs.*

In order not to prolong the foregoing descriptions, a number of important steps in the preparation of the sacs were not emphasized or discussed. Most important among these steps are those that determine permeability. This subject was treated at some length in the former paper on collodion sacs, and will not be reiterated here. But attention may be called to several facts which have more recently been noted.

*Composition of the Collodion.*—We have used a commercial collodion,[7] evaporated in a partial vacuum until thick enough to give a strong membrane with a single coating of the molds. A sufficiently high degree of permeability is conferred upon the membranes by immersion in 95 per cent alcohol, as recommended by Brown.[8] Eggerth,[9] who credits Malfitano with a prior observation, has found that the relative amounts of alcohol and ether in the collodion determine permeability, and has described a series of membranes of graded permeability produced by varying their alcoholic content. Eggerth also found that the addition of 10 to 30 per cent of lactic acid to the collodion greatly increases the permeability of the resulting membranes. These membranes are transparent, tough, and elastic, and of such a high degree of permeability that they readily pass the proteins of serum and ascitic fluid. This is a drawback to their use with a tissue medium when a bacterial growth free from foreign protein is desired. By varying the alcoholic content of the collodion, or by the addition of lactic acid, or by immersion of the undried membrane in alcohol solutions of varied concentrations, a wide range of permeabilities may be obtained.

Eggerth observed that alcohol-rich collodions are made more viscous by the addition of water, and that the presence of even traces of moisture causes them slowly to set. We have had the same experience with thick collodion solutions. Only by keeping the collodion dry can a thick solution be maintained in a fluid state.

[7] Squibb's Contractile Collodion, U. S. P.

[8] Brown, W., *Biochem. J.*, 1915, ix, 591.

[9] Eggerth, A. H., *J. Biol. Chem.*, 1921, xlviii, 203.

*Sterilization by Heat.*—The shrinkage and the loss in permeability of collodion membranes when subjected to sterilization in the autoclave were discussed in the former paper. This shrinkage indicates a change in plasticity and cohesion under the influence of heat. Membranes immersed in alcohol are highly plastic and will stretch considerably under pressure without breaking. Thus a sac with a capacity of 45 cc. was stretched to a capacity of 77 cc. by an internal pressure of 12 cm. of 95 per cent alcohol, when its further expansion was checked by the container. On replacement of the alcohol by water, collodion membranes become more rigid, so that water pressures up to the breaking point may be withstood without change in size. If the membranes are heated, however, their plasticity is increased, and the shape that a sac assumes is the result of a balance between the forces of cohesion and the pressure applied to the sac wall. At atmospheric pressure the membrane shrinks progressively as the temperature is raised, but this tendency may be counteracted by the application of pressure within the sac. A slight pressure prevents shrinkage; a greater pressure expands the sac just as it does a sac immersed in alcohol at room temperature. The following experiment illustrates this fact.

*Experiment 1. Effect of Pressure on Heated Collodion Membranes. Sac A, Unheated Control.*—Capacity 54.8 cc. Immersed in water at room temperature under an internal pressure of 30 cm. of water. After 15 minutes at this pressure, the capacity of the sac was still 54.8 cc. showing that the elastic limit of the membrane had not been exceeded.

*Sac B, Heated at Atmospheric Pressure.*—Capacity 59 cc. Immersed in a water bath at 20°C. Temperature raised to boiling over a period of about 9 minutes. Boiled 5 minutes. Capacity after heating 38.5 cc.

*Sac C, Heated under an Internal Pressure of 25 Cm. of Water.*—Immersed in a water bath at 20°C. (with Sac B). Temperature raised to boiling over a period of about 9 minutes. Boiled 5 minutes. Capacity after heating 75.5 cc.

The appearance of these sacs after the experiment is shown in Fig. 3. The relative plasticity of Sacs B and C during the heat treatment is further illustrated by the pear shape they have assumed. Sac A retains the shape of the mold in which it was made.

When a membrane has once been heated, it becomes permanently less plastic and subsequent reheating does not alter its shape and size to a similar extent.

*Experiment 2. Effect of Pressure on Reheated Collodion Membranes.*—Sac A had a capacity before heating of 52.7 cc. This sac was heated at atmospheric pressure and boiled in a water bath for 9 minutes. After heating, its capacity was 38.7 cc. The sac was again heated under an internal pressure of 32 cm. of water, and boiled for 5 minutes. The capacity of the sac was then 44 cc.

Sac B, capacity 53.5 cc., was boiled under a pressure of 32 cm. of water. The capacity of the sac increased to 68.5 cc. Then the sac was boiled for 5 minutes at atmospheric pressure. Its capacity was reduced to 64.5 cc.

If the shrinkage which occurs in the autoclave is undesirable, it may readily be prevented by sterilization under a slight internal pressure produced by filling the glass neck of the sac with water to a predetermined level, or by a preliminary immersion of the sac in boiling water under a similar water pressure. As tested by the rate of filtration of water under 25 cm. pressure, however, very little difference in permeability results from the prevention of shrinkage, or the expansion of the sac during sterilization by heat. Since it has been possible to prepare sacs which are too permeable for our purpose, the loss in permeability which an undried sac undergoes on sterilization has not been a drawback. Excessive shrinkage due to high temperature is to be avoided, however. We autoclave our sacs for 1 hour at 108°C. (5 pounds steam pressure).

*Mode of Use of the Sacs.*—The method of filling the sacs and the various uses to which they may be put hardly need a detailed description. Various applications will suggest themselves to meet individual requirements. The apparatus is convenient for either aerobic or anaerobic cultivation. For aerobes the space around the sac should not be entirely filled with fluid. For anaerobes, all bubbles should be tipped out of this space, and both limbs must be sealed with vaseline, or the cultures placed in an anaerobic jar. The tubes or flasks may be inoculated immediately, but we have found it advisable to incubate them first for 24 to 48 hours, to prove their sterility and to permit dialysis of nutrient materials. During this period anaerobic conditions will develop under a vaseline seal. It may be pointed out in this connection that while a seal of vaseline excludes oxygen, it also prevents the free escape of carbon dioxide from the medium. Retention of carbon dioxide may lead to a more rapid acidification of the medium than would otherwise occur.

*Hydrogen Ion Concentration of the Dialysate.*—In general, the dialysate acquires the hydrogen ion concentration of the medium within the sac. In a well buffered colloidal medium, however, the diffusion of substances which change the hydrogen ion concentration is relatively slow, and consequently the acid produced in the vicinity of a tissue fragment may not be neutralized by a supernatant column of alkaline medium, and an undue proportion of it may diffuse through the sac wall and acidify the dialysate. We have occasionally found it desirable, therefore, to buffer the dialysate or to bring it to a faint alkalinity with sodium hydroxide before inoculation. Phosphate mixtures in the absence of protein appear to be toxic to some bacteria. Peptone broth, when its use is not contraindicated, is an effective buffer and has been used both as a diluent for serum in the medium, and as a dialysate fluid. The addition of dextrose, 1 to 2 per cent, hastens the establishment of anaerobic conditions, and of course increases the nutritive value of the medium for organisms which can utilize it.

*Osmotic Pressure.*—The osmotic pressure developed in the system depends upon the permeability of the sac in relation to the composition of the culture medium. When a medium containing substances in solution that will not pass through the sac is dialyzed, osmotic pressure adjustments are made automatically by imbibition of water and a rise in the level of the medium within the sac. Thus in Fig. 4 the difference in the levels of medium and dialysate represents the osmotic pressure of a mixture of horse serum and dextrose broth dialyzed against distilled water. Imbibition of water may be prevented by filling the sac and its glass neck approximately to the osmotic pressure level, as found by experience with the given medium. The neck of the sac may be filled beyond the pressure level. Then a filtration of fluid from the sac into the surrounding liquid results that may aid the diffusion of nutritive substances. When collodion containing 10 per cent of lactic acid is used, and the sacs are treated with 95 per cent alcohol (also containing lactic acid), their permeability is such that serum proteins pass through freely, and no difference in levels is established (Fig. 5). Sometimes changes in level break a vaseline seal which is to be restored by gentle heating.

*Symbiosis.*—Eggerth[9] found that *Bacillus influenzæ* Pfeiffer could be grown in plain broth in a sac surrounded by a living culture of staph-

ylococcus, streptococcus, or pneumococcus. We have had a similar experience with *Bacterium pneumosintes*. *Bacillus subtilis* was grown in dextrose broth within collodion sacs. After 24 hours the growth was checked and anaerobic conditions were established by the addition of a vaseline seal. *Bacterium pneumosintes* then grew luxuriantly in dextrose broth surrounding the sacs, although it failed to grow in the same medium in control tubes without *Bacillus subtilis*.

It appears, therefore, that the growth-promoting substances, presumably catalytic or enzymatic in nature, which are necessary to the multiplication of microorganisms may diffuse through collodion membranes of high permeability.

*Primary Isolation of Highly Parasitic Organisms.*—What little experience we have had indicates that the Smith-Noguchi ascitic fluid-tissue culture is a more favorable medium for the primary cultivation of highly parasitic organisms than is the dialysate of such a medium obtained with collodion sacs. When growth on an artificial medium is once established, however, subplants of *Treponema pallidum*, *Treponema microdentium*, and *Bacterium pneumosintes* have grown readily in the dialysate fluid. The absence of precipitate from the medium permits the collection of a sediment for antigenic and serological purposes composed of the microbic bodies alone. In the case of *Bacterium pneumosintes*, this has made possible a study of its antigenic character.

## SUMMARY.

A simple and convenient method is described for the preparation and use of collodion sacs for aerobic and anaerobic bacterial cultivation *in vitro*.

The sacs are suitable for the study of various problems in bacterial growth and metabolism. By their use with the Smith-Noguchi tissue medium, certain microorganisms may be grown in the absence of the confusing, antigenic protein precipitate characteristic of cultures containing fresh tissue, and thus the organisms are obtained in a suitable condition for serological study.

## EXPLANATION OF PLATES.

### PLATE 53.

FIG. 1. Solutions of collodion, gelatin, and alcohol in museum jars. Cluster of molds inserted in a rubber stopper which fits the jars. 50 cc. Erlenmeyer flask lined with a gelatin film. Hook for lowering molds into boiling water.

FIG. 2. Rack for draining and drying Erlenmeyer flasks lined with gelatin.

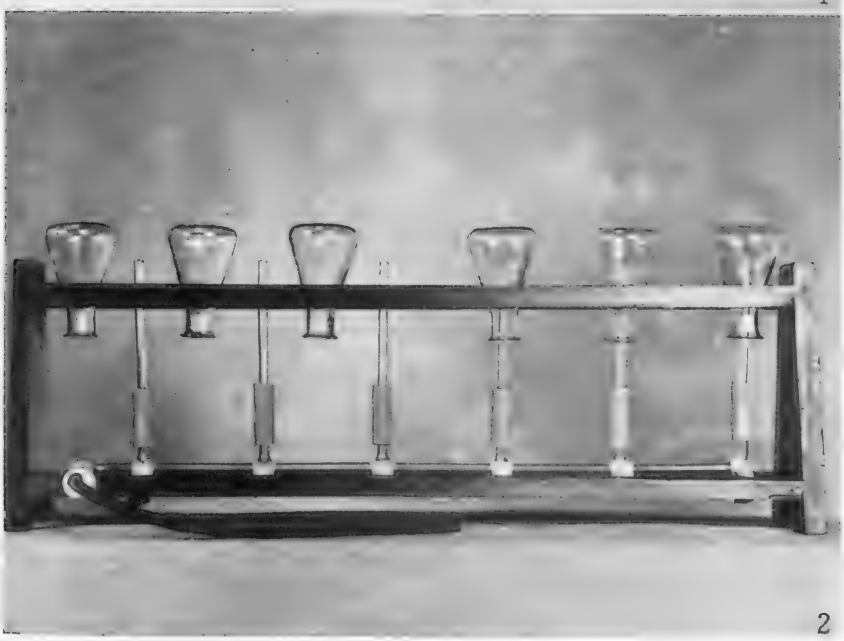
### PLATE 54.

FIG. 3. Sacs described in Experiment 1. Sac A, unheated control; Sac B, heated under atmospheric pressure; Sac C, heated under an internal pressure of 25 cm. of water. Note the pear shape assumed by Sacs B and C.

FIG. 4. Sac system in a V-tube, containing a kidney tissue medium *versus* distilled water. Both liquids are layered with vaseline. The difference in levels indicates the osmotic pressure of a mixture of horse serum and dextrose broth.

FIG. 5. Flask A, a culture of *Treponema pallidum* in the dialysate of an ascitic fluid-dextrose broth-kidney tissue medium. The microorganisms do not cloud the fluid, but grow in feathery gray flocculi at the bottom of the flask. Flask B, a culture of *Bacterium pneumosintes* in the dialysate of a horse serum-dextrose broth-kidney tissue medium. As shown by the faint image of the string in the tube, the fluid is heavily clouded. These two sacs, made with lactic acid collodion, are so permeable that the level of the medium is not raised by osmotic pressure.

1



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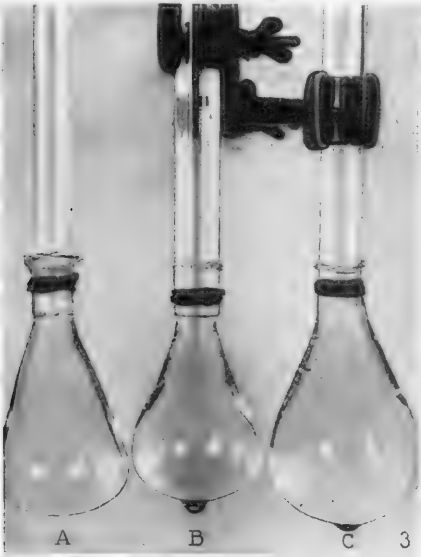
(Gates: Collodion sacs for bacterial cultivation.)

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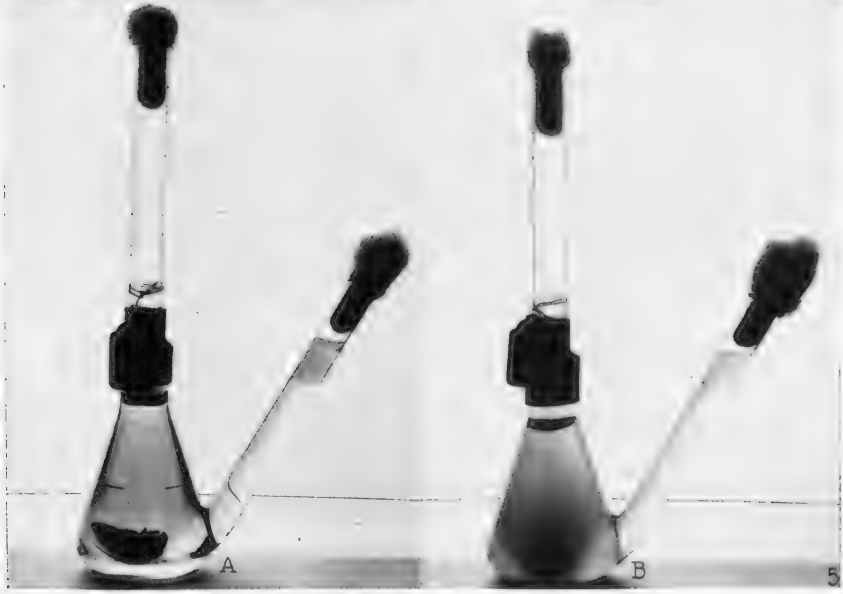


5

(Gates: Collodion sacs for bacterial cultivation.)

# HEAT AND GROWTH-INHIBITING ACTION OF SERUM.

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## INTRODUCTION.

It is known that plasma or serum obtained from an adult animal restrains the growth of a pure culture of homologous fibroblasts.[1] This inhibiting power of plasma or serum is not apparent in very young animals, but manifests itself a few weeks after birth, increases progressively during adult life, and becomes very marked in old age. If it should restrain cell proliferation *in vivo* as much as *in vitro*, its rôle in many physiological and morbid processes would be important. Therefore, an investigation of its nature was begun. The purpose of the experiments described in this article was to study the modifications occurring in the rate of growth of fibroblasts when the serum composing the culture medium had been heated at various temperatures.

## EXPERIMENTAL.

The serum was obtained from the plasma of chickens about 1 year old which had fasted for 24 hours. Its inhibiting action was determined by comparing the rate of growth of fibroblasts in a medium containing 50 per cent serum and in another containing 50 per cent Tyrode solution. The growth index of this serum averaged 0.9, which indicated that the inhibiting power was slight. The serum was used in the preparation of the medium in two different concentrations. When the fibrin of the coagulum was obtained from plasma, the medium was composed of 33.3 per cent plasma, 4 per cent tissue juice, and 62.6 per cent serum. When fibrinogen was used instead of plasma, the medium was made of 20 per cent fibrinogen suspension,[2]

[1] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 599.

[2] Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiii, 641.

50 per cent serum, 27.5 per cent Tyrode solution, and 2.5 per cent tissue juice.

The action of unheated serum was compared with that of heated serum. Serum heated at 56°C. for a period of time varying from ½ to 24 hours became slightly opalescent. When it was heated at 70°C. for ½ hour, a precipitate occurred which was thrown down by centrifugation. The supernatant fluid was transparent, slightly opalescent, and not very viscous. The serum heated at 100°C. coagulated. The fluid which remained around the coagulum was used in the preparation of the medium.

The fibroblasts were taken from stock cultures of a 9 year old strain of connective tissue, and the cultures prepared in the usual manner. They were allowed to grow for 48 hours and the measurements were taken by outlining the original fragment and the new tissue under the projection apparatus and determining the area with the planimeter. The relative increase was calculated.[3]

*1. Action of Serum Heated at 56°C.*—In the first series of experiments, a comparison of the action of sera unheated and heated at 56°C. for 1 hour was made in media containing 33.3 per cent chicken plasma (Tables I and II). The rate of growth of fibroblasts in both media was identical.

In a second series of experiments, 20 per cent fibrinogen suspension was used instead of plasma. Differences in the rate of growth under the influence of heated and unheated sera became apparent (Tables III to VIII). The time during which the serum was kept at the temperature of 56°C. had little influence on its inhibiting action. The rate of growth of fibroblasts in sera heated at 56°C. for ½ to 24 hours was decreased about 15 per cent.

*2. Action of Serum Heated at 70°C.*—In the first series of experiments, the action of serum heated at 70°C. for ½ hour was compared with that of serum heated at 56°C. (Table IX). The rate of growth in the serum heated at 70°C. was 22 per cent slower than in the serum heated at 56°C.

In a second series of experiments, the action of sera unheated and heated at 70°C. was compared (Table X). The growth was found to

[3] Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 231.

TABLE I.

*Rate of Growth of Fibroblasts in Plasma and Serum, Unheated and Heated at 56°C.*

| Experiment No. | Culture No. | Width of ring. Serum 62.6 per cent. Unheated. | Width of ring. Serum 62.6 per cent. Heated. |
|---|---|---|---|
| 1 | 23191 | 5.2 | 4.9 |
| 2 | 23192 | 4.9 | 5.1 |
| 3 | 23193 | 4.6 | 4.7 |
| Average | | 4.9 | 4.9 |
| 4 | 1185–1, 1185–2 | 5.0 | 4.5 |
| 5 | 1185–3, 1185–4 | 4.0 | 4.0 |
| Average | | 4.5 | 4.25 |
| 6 | 23194 | 5.0 | 4.5 |
| 7 | 23195 | 4.0 | 4.5 |
| 8 | 23196 | 5.0 | 5.0 |
| Average | | 4.7 | 4.7 |

The serum used in the first three experiments was heated at 56°C. for 1 hour, in the remaining five experiments, for 2 hours. The rate of growth is expressed by the width of the ring of new tissue.

TABLE II.

*Rate of Growth of Fibroblasts in Plasma and Serum, Unheated and Heated at 56°C.*

| Experiment No. | Culture No. | Relative increase. Serum 62.6 per cent. Unheated. | Relative increase. Serum 62.6 per cent. Heated. | Ratio: $\frac{\text{Heated serum}}{\text{Unheated serum}}$. |
|---|---|---|---|---|
| 1 | 23191 | 4.08 | 4.02 | 0.99 |
| 2 | 23192 | 3.76 | 3.91 | 1.04 |
| 3 | 23193 | 3.46 | 4.05 | 1.17 |
| Average | | | | 1.07 |
| 4 | 23194 | 3.85 | 3.61 | 0.94 |
| 5 | 23196 | 3.82 | 4.07 | 1.07 |
| Average | | | | 1.00 |

In the first group of experiments, the serum was heated at 56°C. for 1 hour, in the second group, for 2 hours. The rate of growth is expressed by the relative increase of the tissue.

TABLE III.

*Rate of Growth of Fibroblasts in Serum, Unheated and Heated at 56°C. for ½ Hr.*

| Experiment No. | Culture No. | Relative increase. Serum 50 per cent. Unheated. | Relative increase. Serum 50 per cent. Heated. | Ratio: $\frac{\text{Heated serum}}{\text{Unheated serum}}$. |
|---|---|---|---|---|
| 1 | 20936 | 3.36 | 2.72 | 0.80 |
| 2 | 20937 | 2.13 | 1.62 | 0.76 |
| 3 | 20938 | 3.50 | 2.68 | 0.77 |
| 4 | 21491 | 5.06 | 4.87 | 0.96 |
| 5 | 21492 | 3.43 | 2.93 | 0.85 |
| 6 | 21493 | 4.80 | 3.43 | 0.71 |
| 7 | 21450 | 2.76 | 2.51 | 0.91 |
| 8 | 21841 | 2.90 | 2.20 | 0.76 |
| 9 | 21842 | 2.60 | 2.40 | 0.92 |
| 10 | 21843 | 2.80 | 2.30 | 0.82 |
| 11 | 21844 | 2.40 | 2.40 | 1.00 |
| 12 | 23110 | 2.16 | 1.89 | 0.88 |
| Average | | 3.16 | 2.66 | 0.85 |

TABLE IV.

*Rate of Growth of Fibroblasts in Serum, Unheated and Heated at 56°C. for 1 Hr.*

| Experiment No. | Culture No. | Relative increase. Serum 50 per cent. Unheated. | Relative increase. Serum 50 per cent. Heated. | Ratio: $\frac{\text{Heated serum}}{\text{Unheated serum}}$. |
|---|---|---|---|---|
| 1 | 23164 | 3.72 | 3.42 | 0.92 |
| 2 | 23165 | 3.73 | 3.37 | 0.90 |
| 3 | 23166 | 2.91 | 2.91 | 1.00 |
| 4 | 23167 | 2.48 | 2.30 | 0.93 |
| 5 | 23226 | 3.55 | 4.00 | 1.12 |
| 6 | 23227 | 4.35 | 4.47 | 1.03 |
| 7 | 23246 | 2.28 | 1.81 | 0.79 |
| 8 | 23248 | 2.74 | 2.00 | 0.73 |
| 9 | 23249 | 2.21 | 1.85 | 0.84 |
| Average | | | | 0.92 |
| 10 | 23477 | 5.12 | 4.64 | 0.91 |
| 11 | 23478 | 3.92 | 3.55 | 0.91 |
| 12 | 23479 | 3.80 | 3.12 | 0.82 |
| 13 | 23944 | 2.81 | 2.22 | 0.79 |
| 14 | 23945 | 3.20 | 2.65 | 0.83 |
| 15 | 23946 | 3.14 | 2.56 | 0.82 |
| Average | | | | 0.85 |

In the first nine experiments, 5 per cent embryo juice was used, and in the remaining six experiments, 2.5 per cent embryo juice.

TABLE V.

*Rate of Growth of Fibroblasts in Serum, Unheated and Heated at 56°C. for 2 Hrs.*

| Experiment No. | Culture No. | Relative increase. Serum 50 per cent. Unheated. | Relative increase. Serum 50 per cent. Heated. | Ratio: $\frac{\text{Heated serum}}{\text{Unheated serum}}$. |
|---|---|---|---|---|
| 1 | 23168 | 3.12 | 2.77 | 0.89 |
| 2 | 23169 | 3.00 | 2.74 | 0.91 |
| 3 | 23170 | 3.19 | 2.67 | 0.84 |
| 4 | 23171 | 3.10 | 2.83 | 0.91 |
| 5 | 23228 | 3.50 | 3.22 | 0.92 |
| 6 | 23229 | 3.93 | 3.80 | 0.97 |
| 7 | 23251 | 3.24 | 2.85 | 0.88 |
| 8 | 23252 | 2.55 | 2.33 | 0.91 |
| 9 | 23253 | 3.32 | 3.00 | 0.90 |
| Average | | | | 0.90 |

In the first two experiments, 5 per cent embryo juice was used, and in the remaining seven experiments, 2.5 per cent embryo juice.

TABLE VI.

*Rate of Growth of Fibroblasts in Serum, Unheated and Heated at 56°C. for 6 Hrs.*

| Experiment No. | Culture No. | Relative increase. Serum 50 per cent. Unheated. | Relative increase. Serum 50 per cent. Heated. | Ratio: $\frac{\text{Heated serum}}{\text{Unheated serum}}$. |
|---|---|---|---|---|
| 1 | 23288 | 3.21 | 2.70 | 0.84 |
| 2 | 23289 | 4.21 | 3.20 | 0.76 |
| 3 | 23290 | 4.05 | 3.21 | 0.79 |
| Average | | | | 0.80 |

TABLE VII.

*Rate of Growth of Fibroblasts in Serum, Unheated and Heated at 56°C. for 12 Hrs.*

| Experiment No. | Culture No. | Relative increase. Serum 50 per cent. Unheated. | Relative increase. Serum 50 per cent. Heated. | Ratio: $\frac{\text{Heated serum}}{\text{Unheated serum}}$. |
|---|---|---|---|---|
| 1 | 23291 | 3.50 | 2.78 | 0.79 |
| 2 | 23292 | 3.70 | 3.11 | 0.84 |
| 3 | 23293 | 3.67 | 3.22 | 0.88 |
| Average | | | | 0.84 |

TABLE VIII.

*Rate of Growth of Fibroblasts in Serum, Unheated and Heated at 56°C. for 24 Hrs.*

| Experiment No. | Culture No. | Relative increase. Serum 50 per cent. Unheated. | Relative increase. Serum 50 per cent. Heated. | Ratio: $\frac{\text{Heated serum}}{\text{Unheated serum}}$. |
|---|---|---|---|---|
| 1 | 23440 | 3.70 | 3.08 | 0.83 |
| 2 | 23441 | 3.58 | 2.93 | 0.82 |
| 3 | 23442 | 3.87 | 3.03 | 0.78 |
| 4 | 23334 | 3.07 | 2.90 | 0.94 |
| 5 | 23335 | 3.26 | 3.15 | 0.96 |
| 6 | 23336 | 3.48 | 2.90 | 0.83 |
| Average | | | | 0.86 |

TABLE IX.

*Rate of Growth of Fibroblasts in Serum Heated at 56°C. for ½ Hr. and at 70°C. for ½ Hr.*

| Experiment No. | Culture No. | Relative increase. Serum 50 per cent. Heated at 56°C. | Relative increase. Serum 50 per cent. Heated at 70°C. | Ratio: $\frac{\text{Serum heated at 70°C.}}{\text{Serum heated at 56°C.}}$. |
|---|---|---|---|---|
| 1 | 23812 | 2.70 | 1.98 | 0.73 |
| 2 | 23813 | 2.33 | 2.00 | 0.86 |
| 3 | 23814 | 3.06 | 2.56 | 0.84 |
| 4 | 23846 | 2.70 | 2.18 | 0.81 |
| 5 | 23847 | 2.56 | 1.92 | 0.75 |
| 6 | 23848 | 2.76 | 2.08 | 0.75 |
| 7 | 23886 | 3.26 | 2.76 | 0.85 |
| 8 | 23887 | 2.67 | 2.37 | 0.89 |
| 9 | 23888 | 3.00 | 2.20 | 0.73 |
| 10 | 23941 | 3.40 | 2.44 | 0.72 |
| 11 | 23942 | 3.40 | 2.43 | 0.71 |
| 12 | 23943 | 3.78 | 2.80 | 0.74 |
| 13 | 23947 | 2.39 | 1.74 | 0.73 |
| 14 | 23948 | 2.33 | 1.81 | 0.78 |
| 15 | 23949 | 2.84 | 2.28 | 0.80 |
| Average | | | | 0.78 |

TABLE X.

*Rate of Growth of Fibroblasts in Serum, Unheated and Heated at 70°C. for ½ Hr.*

| Experiment No. | Culture No. | Relative increase. Serum 50 per cent. Unheated. | Relative increase. Serum 50 per cent. Heated. | Ratio: $\frac{\text{Heated serum}}{\text{Unheated serum}}$. |
|---|---|---|---|---|
| 1 | 23337 | 3.20 | 2.28 | 0.71 |
| 2 | 23338 | 4.33 | 3.14 | 0.73 |
| 3 | 23339 | 4.24 | 2.69 | 0.63 |
| 4 | 23443 | 3.91 | 2.08 | 0.53 |
| 5 | 23444 | 3.43 | 1.69 | 0.49 |
| 6 | 23445 | 3.95 | 1.69 | 0.43 |
| 7 | 23709 | 3.62 | 2.25 | 0.62 |
| 8 | 23710 | 3.90 | 2.57 | 0.66 |
| 9 | 23711 | 3.90 | 2.60 | 0.67 |
| 10 | 23679 | 2.38 | 1.94 | 0.82 |
| 11 | 23680 | 2.21 | 1.80 | 0.81 |
| 12 | 23681 | 3.88 | 2.93 | 0.76 |
| Average | | | | 0.66 |

be 34 per cent slower in heated than in unheated serum. Both series of experiments showed almost identical results. It must be observed also (Tables IX and X) that in both series the values for the relative increase of the tissues in serum heated at 70°C. are about similar. The consistency of the results demonstrates that there were no technical errors.

TABLE XI.

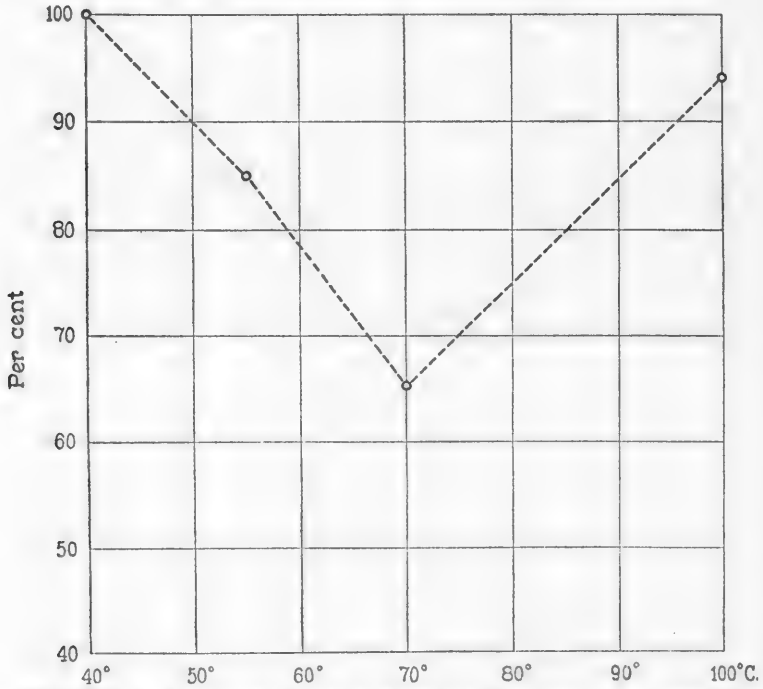
*Rate of Growth of Fibroblasts in Serum Heated at 100°C. for 10 Min. and at 70°C. for ½ Hr.*

| Experiment No. | Culture No. | Relative increase. Serum 50 per cent. Heated at 100°C. | Relative increase. Serum 50 per cent. Heated at 70°C. | Ratio: $\frac{\text{Heated at 70°C.}}{\text{Heated at 100°C.}}$. |
|---|---|---|---|---|
| 1 | 23889 | 3.35 | 2.30 | 0.69 |
| 2 | 23890 | 2.60 | 1.90 | 0.73 |
| 3 | 23891 | 3.26 | 2.55 | 0.78 |
| Average | | | | 0.73 |

TABLE XII.

*Rate of Growth of Fibroblasts in Serum, Unheated and Heated at 100°C. for 10 Min.*

| Experiment No. | Culture No. | Relative increase. Serum 50 per cent. Unheated. | Relative increase. Serum 50 per cent. Heated. | Ratio: $\frac{\text{Heated serum}}{\text{Unheated serum}}$. |
|---|---|---|---|---|
| 1 | 23632 | 3.12 | 3.06 | 0.98 |
| 2 | 23633 | 3.43 | 3.01 | 0.88 |
| 3 | 23634 | 3.19 | 3.02 | 0.95 |
| 4 | 23635 | 2.88 | 2.85 | 0.99 |
| 5 | 23654 | 2.91 | 2.87 | 0.99 |
| 6 | 23655 | 3.05 | 3.44 | 1.13 |
| 7 | 23656 | 3.32 | 3.14 | 0.94 |
| 8 | 23657 | 3.30 | 2.90 | 0.88 |
| 9 | 23849 | 4.03 | 3.25 | 0.81 |
| 10 | 23850 | 3.16 | 2.74 | 0.87 |
| 11 | 23851 | 3.81 | 3.56 | 0.93 |
| Average ..... | | | | 0.94 |




TEXT-FIG. 1. Variations in the rate of growth of fibroblasts in function of the temperature at which the serum was heated. The rate of growth of fibroblasts in unheated serum was considered to be equal to 100 per cent.

*3. Action of Serum Heated at 100°C.*—The fluid remaining after coagulation was compared with serum heated at 70°C. and with unheated serum. The figures show that the rate of growth in serum heated at 100°C. was 27 per cent faster than in serum heated at 70°C.; that is, about equal to the growth rate in unheated serum (Table XI). The experiments in which a comparison was made between the respective actions of unheated serum and serum heated at 100°C. confirmed these results (Table XII). The amount of growth was practically identical in both unheated serum and serum heated at 100°C. It must be remembered that differences equal to or smaller than 10 per cent may be due to experimental errors.

A curve was plotted expressing the variations of the rate of growth of the fibroblasts in function of the temperature at which the serum had been heated (Text-fig. 1). It shows that the rate of cell proliferation decreased after the serum had been heated at 56°C., and became still slower after it had been heated at 70°C. A marked increase in the rate of growth took place after the serum had been heated at 100°C.

## DISCUSSION.

The results obtained in the preceding experiments may be summarized as follows:

The action of heat at 56°C. increased by 15 per cent the inhibiting action of serum obtained from young adult chickens on the proliferation of fibroblasts. The action of heat at 70°C. increased the inhibiting action by 34 per cent. When the serum had been heated at 100°C., its inhibiting action became about equal to that of non-heated serum. Therefore, heated serum contained a factor which markedly inhibited the growth of fibroblasts and which developed at, or resisted a temperature of 70°C.

These experiments confirmed the results obtained by Ingebrigtsen[4] in his study of the growth of guinea pig bone marrow in homologous serum unheated and heated at 56°C. The growth of bone marrow was found to be more extensive in unheated than in heated serum. The differences in the action of both sera were more striking than in

[4] Ingebrigtsen, R., *J. Exp. Med.*, 1912, xv, 397.

our experiments. This was due to the fact that Ingebrigtsen used a medium containing a very large amount of serum and observed lymphocytes instead of fibroblasts.

The increase of the inhibiting power of serum after it had been heated at 56° and 70°C. may be considered as due to the production, under the influence of heat, of a change which renders the serum more toxic for the homologous fibroblasts. But it may also be attributed to the destruction of substances presenting the same heat resistance as complement and amboceptor, and partly protecting the cells against the inhibiting action of a third substance resisting heat at 70°C. Serum modified by heat acts in an opposite manner on heterologous tissues. Heated serum is a better culture medium for heterologous cells than unheated serum, as has already been shown by Ingebrigtsen.[4] We have found lately that the inhibiting action of dog, rabbit, and cat serum, heated at 56° and 66°C., on the rate of multiplication of fibroblasts is very much decreased. It seems that the factors which protect the organism against foreign cells and bacteria might also oppose the growth-inhibiting factor of serum and allow the cells to display a greater activity.

## CONCLUSIONS.

The inhibiting action of homologous serum on the proliferation of fibroblasts *in vitro* was increased after the serum had been heated at 56° and 70°C. This action decreased after the serum had been heated at 100°C.

# CICATRIZATION OF WOUNDS.

## XIII. The Temperature Coefficient.

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(Received for publication, January 11, 1922.)

### INTRODUCTION.

Cicatrization is a complex phenomenon which probably requires the coordinated activity of many factors. Although it may be expressed by a simple formula,[1] its mechanism involves several different agencies. The formation of granulating tissue during the latent period,[2] its contraction, the mobilization and proliferation of the epithelium, and the wandering of the latter on the surface of the granulations are themselves intricate processes, which may be governed by physical as well as chemical changes. Alterations of the viscosity of the surface tension of the fluids and the anatomical structures may play as important a rôle as chemical transformations. In order to ascertain whether physical or chemical changes are more especially involved, the value of the temperature coefficient of the phenomenon was measured.

### EXPERIMENTAL.

The experiments were performed on alligators because these animals may be kept as well at a temperature of 38° as at 23°C. Two young alligators were used, their weights being respectively 309 and 72 gm. A rectangular flap of skin on the ventral surface of the body was resected with a sharp knife and the outline of the wound traced with India ink upon a piece of cellophane placed over the surface. The tracings were transferred to paper and the areas measured with a

[1] du Noüy, P. L., *J. Exp. Med.*, 1919, xxix, 329.

[2] Carrel, A., *J. Exp. Med.*, 1921, xxxiv, 425.

planimeter. The animals were placed in a room having a temperature of 38°C. until the wounds had healed. Several days later a second resection of skin was made in a different area on the ventral surface of the body of each animal, as nearly as possible the same size as the first. This was done by transferring the outline of the previous tracing to the skin surface and then excising the flap. The animals were placed in a room, the temperature of which averaged 23°C.

The wounds could not be protected by a dressing. They were necessarily slightly infected, and a membrane or scab formed at the surface. Under this covering, cicatrization was taking place, but its progress could not be followed closely. To overcome this hindrance to precise observation, the edges of the scab were gently and progressively loosened with the point of a knife and a pair of small thumb forceps, and then the extent of the formation of the epithelial layer under the scab could be seen. The membrane was easily loosened from the portion of the wound which had already healed, while the

TABLE I.

| Experiment No. | Weight of animal. | Temperature. | Area of wound. | Length of time required for cicatrization. | Temperature coefficient for 10°C. |
|---|---|---|---|---|---|
| | *gm.* | *°C.* | *sq. cm.* | *days* | |
| 1 | 309 | 38 | 1.2 | 11 | 2.36 |
| | 314 | 23 | 1.3 | 29 | |
| 2 | 72 | 38 | 0.4 | 8 | 1.88 |
| | 75 | 23 | 0.5 | 19 | |

scab proved adherent wherever epithelization had not occurred. If an attempt were made to remove the protecting membrane, a slight hemorrhage started. As such a traumatism delays the process of healing, observations had to be made with the greatest care. Errors of 1 or 2 days in the time of cicatrization may have occurred on account of the technical difficulties. Two experiments were made on each animal, one at a temperature of 38°C., and the other at 23°C. (Table I).

In the first experiment, the temperature coefficient obtained, $\frac{K_1}{K_0}$, is equal to 2.47, and in the second to 1.90, for a difference of

15°C., between 38° and 23°C. The difference between the values of these two coefficients may be attributed to experimental errors, but is not of a greater magnitude than those observed in similar phenomena. Their value for a range of 10°C. (between 20° and 30°C.) was obtained by the formula quoted by Robertson:[3]

$$\frac{K_1}{K_o} = e^{\frac{\mu}{2}\left(\frac{T_1 - T_o}{T_1 T_o}\right)}$$

In the first case, it is equal to 2.36, and in the second to 1.88 for the same interval. The mean value is 2.12.

## DISCUSSION AND CONCLUSIONS.

For a rise of temperature of 10°C., the rate of cicatrization was increased about twofold. This result could be expected, since wound healing is closely related to the phenomenon of growth and regeneration. It is classical that changes in temperature affect the metabolism[4] and the development of certain organisms[5] in the same manner as a chemical reaction. In spite of the complexity of the factors which bring about the cicatrization of a wound, it appears that the velocity of the phenomenon depends on the rate at which certain chemical changes take place.

[3] Robertson, T. B., Principles of biochemistry, for students of medicine, agriculture and related sciences, Philadelphia, 1920, 419.

[4] Krogh, A., *Z. allg. Physiol.*, 1914, xvi, 178.

[5] Hertwig, O., *Arch. mikr. Anat.*, 1898, li, 319. Loeb, J., and Northrop, J. H., *Proc. Nat. Acad. Sc.*, 1917, iii, 384. Moore, A. R., *Arch. Entwcklngsmechn.* 1910, xxix, 146.

# ACTION OF ANTIGEN ON FIBROBLASTS IN VITRO.

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It is known that tissue cells cultivated outside of the organism are able to produce antibodies against a given antigen when it is added to the medium. Goat red blood corpuscles, added to the medium in which guinea pig bone marrow was cultivated, determined the production of hemolysins by the guinea pig cells.[1] In the experiments described in this paper, the action of an antigen on the rate of proliferation of fibroblasts was investigated. An attempt was made to learn whether one part of a fragment of culture would grow at the same rate as the other part, when a small amount of a foreign protein was added to the medium for a long period of time; and whether a change in their respective rates of growth would occur if the fibroblasts cultivated in homogenic plasma, and in the same plasma containing a small amount of a heterogenic protein, were transferred into a medium containing this latter protein under a high contentration.

## *Technique.*

The tissues used in these experiments were taken from a 9 year old strain of fibroblasts.[2] The cultures were prepared in the usual way, incubated for 48 hours, measured by projection of the area of growth, and transferred to a fresh medium. The foreign protein used was human ascitic fluid; in a few experiments dog serum was used. The culture which was selected for the experiment was divided into two equal parts. One of the fragments was placed in a medium consisting of equal volumes of chicken plasma and fresh chick embryo juice. The other was placed in a medium containing equal volumes of plasma

[1] Carrel, A., and Ingebrigtsen, R., *J. Exp. Med.*, 1912, xv, 287.

[2] Ebeling, A. H., *J. Exp. Med.*, 1919, xxx, 531. Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 317.

and embryonic tissue juice to which had previously been added about 14 per cent of ascitic fluid, making the percentage of foreign protein in the total culture medium about 7 per cent. Care was taken to avoid contaminating the control culture by instruments, knives, and needles which had been in contact with the foreign protein. When transferred to a new medium, each of the two cultures was divided. One half was used for continuing the experiment, and the other half was discarded, unless this part was used as a test to see how the cultures would act when exposed to the foreign protein in a high concentration.

## RESULTS.

The rate of growth of the two strains of a complete experiment of nineteen passages during a period of about 40 days is recorded in Table I and Text-fig. 1. At the beginning, the rate of growth of both cultures showed a slight difference. The culture to which the small amount of ascitic fluid was added nearly always showed a slightly higher rate of growth than the control culture. This might be due to secondary causes. The fluctuations of the rate of proliferation are due, to some extent, to the influence of slight changes in temperature of the incubator and similar periodical causes. When the control showed a high rate of growth, the experiment showed a lower rate, and *vice versa*, but after about eight or ten passages both cultures showed fluctuations in the same direction. As a whole, no marked differences between the two rates of growth seem to take place.

When an attempt was made to see how both strains would act when exposed to a high concentration of the protein used for immunization, subcultures were made of the control (non-immunized strain) and of the immunized strain, and transferred into a medium composed of plasma, embryonic tissue juice, and ascitic fluid. The amount of ascitic fluid in the subcultures was 50 per cent or more, and exerted a marked inhibiting influence on the rate of growth of a normal culture. The rate of growth of the control cultures which had not been exposed to ascitic fluid decreased markedly and was even followed by death (Text-fig. 2). At Passages 7, 10, 12, 14, and 17, half the tissue fragment was cultivated in a medium containing about 50 per cent of ascitic fluid, while the other half was used for the continuation of the

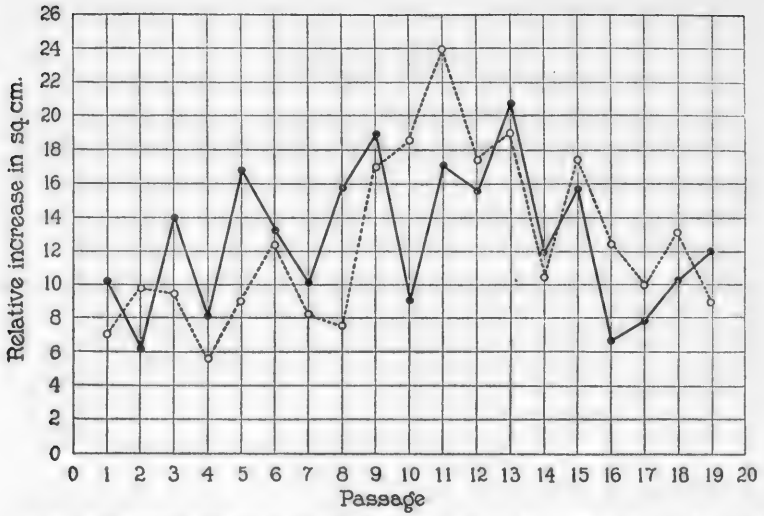
strain. The dots *A*, *B*, *C*, *D*, and *E* represent the rate of growth of these subcultures in 48 hours (Text-fig. 2.) It is shown that 50 per cent ascitic fluid has a marked inhibiting influence on the rate of

TABLE I.

*Rate of Growth of the Immunized and Non-Immunized Strains of Fibroblasts for a Period of Nineteen Passages.*

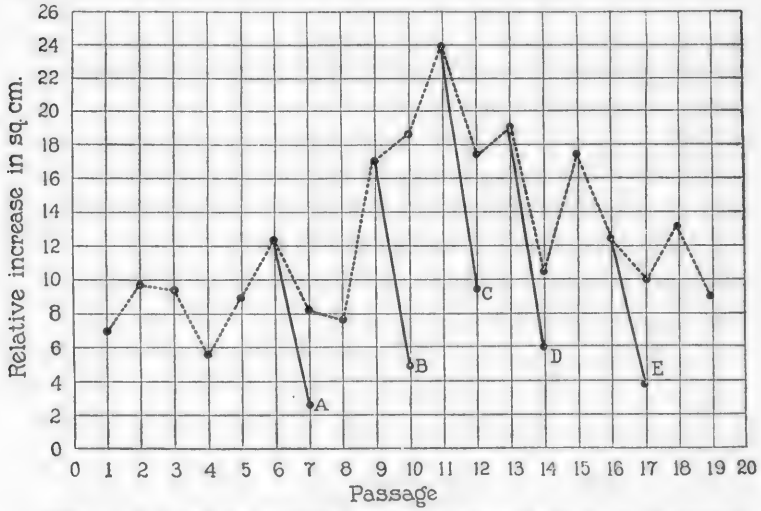
| Culture No. | Passage No. | Relative increase. | | Relative increase of subcultures in 50 per cent ascitic fluid. | | Ratio $\left(\frac{E}{C}\right)$ of subcultures. |
|---|---|---|---|---|---|---|
| | | Control; homogenic medium. | Experiment; homogenic medium with 7 per cent ascitic fluid. | Control. | Experiment. | |
| 537–1, 537–2 | 1 | 7.0 | 10.2 | | | |
| 549, 550 | 2 | 9.8 | 6.2 | | | |
| 559, 560–1 | 3 | 9.4 | 14.0 | | | |
| 571, 572 | 4 | 5.6 | 8.2 | | | |
| 585, 586 | 5 | 9.0 | 16.8 | | | |
| 599, 600 | 6 | 12.4 | 13.4 | | | |
| 610–1, 610–2<br>611–1, 611–2 | 7 | 8.2 | 10.2 | 2.8 | 13.8 | 4.9 |
| 623, 624 | 8 | 7.6 | 15.8 | | | |
| 642, 643 | 9 | 17.0 | 19.0 | | | |
| 659–1, 659–2<br>660–1, 660–2 | 10 | 18.6 | 9.2 | 4.6 | 16.2 | 3.5 |
| 672, 673 | 11 | 23.9 | 17.2 | | | |
| 686–1, 686–2<br>687–1, 687–2 | 12 | 17.4 | 15.6 | 9.4 | 23.2 | 2.4 |
| 702, 703 | 13 | 19.0 | 20.8 | | | |
| 718–1, 718–2<br>719–1, 719–2 | 14 | 10.4 | 12.0 | 6.0 | 23.0 | 3.4 |
| 733, 734 | 15 | 17.4 | 15.8 | | | |
| 749, 750 | 16 | 12.4 | 6.8 | | | |
| 760–1, 760–2<br>761–1, 761–2 | 17 | 10.0 | 8.0 | 3.8 | 8.4 | 4.2 |
| 775, 776 | 18 | 13.2 | 10.4 | | | |
| 799, 800 | 19 | 9.0 | 12.2 | | | |

growth of a normal culture of fibroblasts. The rate of growth of the fibroblasts in a culture medium which always contained about 7 per cent ascitic fluid is shown in Text-fig. 3. At Passages 7, 10, 12, 14, and 17, subcultures were made in a medium containing about 50 per

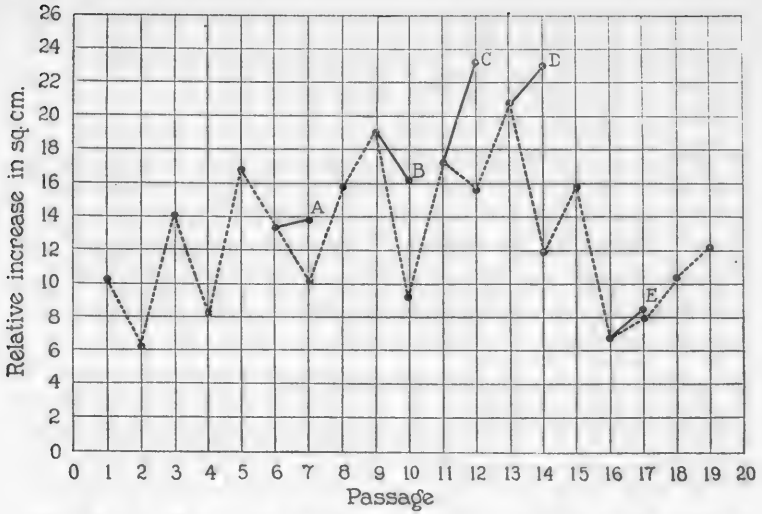



In all the figures the ordinates represent the relative increase in square centimeters and the abscissæ the number of passages at 48 hour intervals.

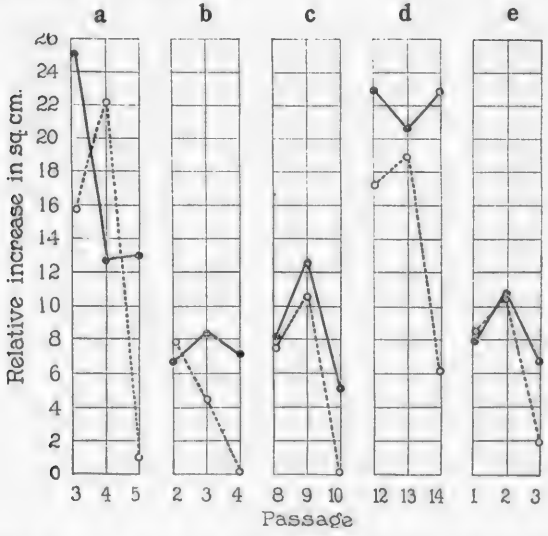
TEXT-FIG. 1. The rate of growth of two cultures of fibroblasts, the control (dotted line) cultivated in the usual homogenic culture medium, the other (solid line) in the same medium containing 7 per cent ascitic fluid.




TEXT-FIG. 2. The rate of growth of the non-immunized strain and subcultures. The dotted line represents the rate of growth of fibroblasts in homogenic culture medium. The solid lines (*A*, *B*, *C*, *D*, and *E*) represent the rate of growth of the subcultures of the non-immunized strain in a medium containing 50 per cent ascitic fluid.




TEXT-FIG. 3. The rate of growth of the immunized strain and subcultures. The dotted line represents the rate of gowth of fibroblasts in the culture medium containing 7 per cent ascitic fluid. The solid lines (*A*, *B*, *C*, *D*, and *E*) represent the rate of growth of the subcultures of the immunized strain in a medium containing 50 per cent ascitic fluid.




TEXT-FIG. 4, *a* to *e*. (*a* to *d*) The rate of growth of the non-immunized (dotted line) and the immunized (solid line) strain of fibroblasts. Of the three passages shown, the last one represents the rate of growth in the culture medium containing 50 per cent ascitic fluid. (*e*) The rate of growth of two strains of fibroblasts, one (solid line) cultivated in a medium containing 7 per cent dog serum, the other (dotted line) in a medium containing no foreign protein. The last passage shows the growth of both strains in the culture medium containing 50 per cent dog serum.

cent ascitic fluid, and the dots *A*, *B*, *C*, *D*, and *E* indicate the rate of growth. It may be seen that the rate of growth increased markedly instead of decreasing, as in the control cultures. There was always a sharp difference in the response of the two cultures to the same amount of foreign protein. It was certain that one of the strains had become adapted to the presence of the foreign protein, while the other was still subject to its inhibiting influence. The same phenomena were observed in several other experiments and are expressed by Text-fig. 4, *a* to *e*. In Text-fig. 4, *a*, the curve shows the rate of growth during two passages in 7 per cent foreign protein, and after exposure to 50 per cent foreign protein. The non-immunized strain almost died. Its rate of growth decreased from 22 sq. cm. to about 1 sq. cm., while the immunized strain grew at the usual rate, as if nothing had happened. In another experiment (Text-fig. 4, *b*), the non-immunized strain was killed, and the immunized strain not affected at all. The same phenomenon is shown in Text-fig. 4, *c* and *d*.

In a few experiments, dog serum which had had a strong inhibiting influence on the growth of fibroblasts was used as antigen. The result showed the same action as with ascitic fluid (Text-fig. 4, *e*). The immunized strain was cultivated in a medium containing 7 per cent dog serum, and the control in a medium containing no foreign protein. For the final test, both strains were placed in a medium containing 50 per cent dog serum; the rate of growth of the control decreased much more than that of the immunized strain.

## CONCLUSIONS.

It may be concluded that, under the conditions of the experiments:

1. A small amount of foreign protein added to the culture medium does not modify the rate of proliferation of fibroblasts.

2. A large amount of foreign protein added to the culture medium decreases markedly the rate of proliferation of fibroblasts cultivated previously in homogenic medium, while it does not decrease the rate of proliferation of fibroblasts cultivated previously in the presence of a small amount of the foreign protein.

3. Fibroblasts *in vitro* respond to the presence of an antigen in the culture medium by becoming immunized against its action.

# THE VASELINE TUBE AND SYRINGE METHOD OF MICRO GAS ANALYSIS OF BACTERIAL CULTURES.

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*(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)*

Plates 55 to 57.

(Received for publication, January 9, 1922.)

The methods to be described have been evolved not so much because of dissatisfaction with results which could be obtained by methods previously used but rather for reasons of greater convenience and economy. It is for these reasons that the Smith fermentation tube has continued in use long after its limitations have been recognized. The Smith tube was never recommended as a means for accurate study of the gas-forming function of bacteria. It was recommended as an instrument for collecting and approximately measuring and analyzing the gases which are given off by certain cultures when grown in this tube. The result of such measurement and analysis was found to be a means of distinguishing certain broad groups of bacteria. The gas was produced under the conditions imposed by the tube and the gas formula $\left(\frac{H_2}{CO_2}\right)$ was understood to be valid for those conditions only. Critics of the Smith fermentation tube should not lose sight of these facts.

More efficient and more elaborate forms of apparatus have been devised by various authors with a different purpose in view, namely to collect all the gas which a culture forms, to measure both that given off from the medium and that held by the medium, and to make very accurate analyses of these gases. I refer particularly to the apparatus described by Keyes (1909), Frieber (1913, *b*), Rogers, Clark and Davis (1914), and Wolf and Harris (1917). These methods are much better adapted for the careful study of the gas-forming activity of a

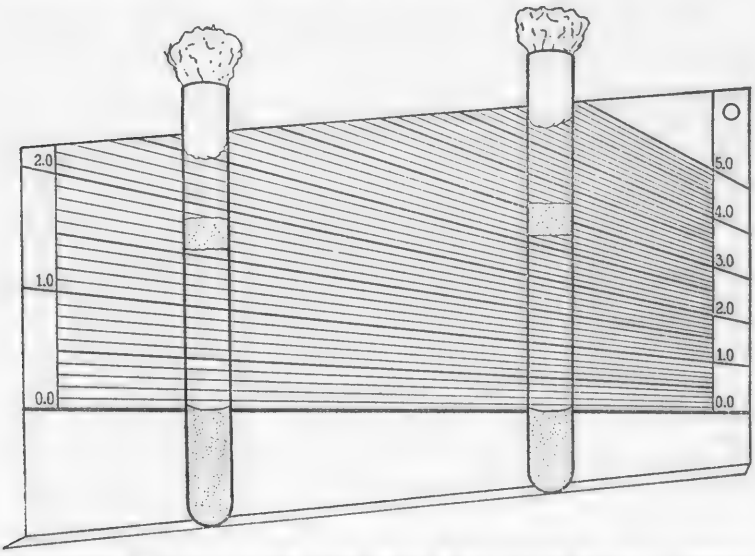
few cultures than for the comparative study of a large number of strains. Doubtless the apparatus described by Van Slyke and Stadie (1921) could be used for bacterial cultures and should yield more accurate results than the syringe. However, it is believed that the method to be described retains the simplicity of the Smith tube, at least something of the greater accuracy of the more elaborate methods, and some advantages for the bacteriologist possessed by none of the older methods.

During a study of anaerobic bacteria still in progress, use was made of small amounts of media covered by vaseline in test-tubes. It was noticed that as gas was formed by a culture the vaseline was forced up the tube, the vaseline plug remaining perfectly intact and acting as a self-lubricating piston at the temperature of the room or incubator. We have continued to use this form of culture tube in the manner to be described.

*Measurement of Gas above the Medium.*

A record of the amount of gas present above the medium may be made at any time by marking with a wax pencil on the side of the tube the lower level of the vaseline plug. To measure the amount of gas in the tube a device somewhat like the Frost gasometer for the Smith fermentation tube is used. The gasometer shown in Text-fig. 1 is made of sheet tin or brass. The lower edge is turned at a right angle towards the observer so that a shelf is formed on which rests the bottom of the tube being examined. The base-line of the graduations ruled on the surface of the gasometer is shown as a double line marked 0.0 and placed diagonally with reference to the shelf and above it. The culture tube is pushed along the shelf until the meniscus of the culture medium rests at the base-line. The graduations above the base-line radiate from a point on the base-line at some distance to the right of the figure so that when the tube cuts the base-line vertically the amount of gas between the medium and the vaseline plug can be read as volumes and tenths of a volume of the amount of medium present in the tube, or, if multiplied by 100, as volumes per cent. If the culture produces sufficient gas to force the vaseline up nearly to the cotton plug the amount of gas is recorded, then by directing the flame of a micro burner against the side of the tube the vaseline is

melted and allowed to drop down onto the surface of the medium and to solidify again. This may be done repeatedly in which case the total gas formed above the medium is obtained by addition of the measurements recorded before each melting of the vaseline plug.




TEXT-FIG. 1. The gasometer with two tubes in position for the measurement of the gas above the medium in terms of the volume of medium employed.

*Determination of $CO_2$ in the Gas above the Medium.*

By means of a Luer tuberculin syringe with a long fine needle[1] attached by means of a short length of capillary rubber tubing a sample of gas may be withdrawn and the $CO_2$ determined in the syringe. The technique and necessary equipment are illustrated in Figs. 1 and 2. The various steps of the technique are as follows:

[1] The needle used was Gauge 20 and 6 inches long, specially made for us by Becton, Dickinson and Co., Rutherford, N. J. There is also needed a small adapter such as that listed by Arthur H. Thomas Co., No. 9419, for 606 Hose End to Luer Slip Needles.

1. Rinse the syringe and needle with dilute acid which is then expelled as completely as possible by forcing air into and out of the syringe a number of times. Push the syringe plunger in as far as it will go or, better, to an exact reading at the lower end of the scale on the barrel of the syringe.

2. Dry and sterilize the needle by passing it through the flame a number of times.

3. With the usual precautions to maintain sterility draw the cotton plug from the culture tube and replace it with the needle held between the cotton plug and the side of the tube.

4. Push the needle down until the point passes through the vaseline plug and well into the zone of gas.

5. With the syringe held as shown in Fig. 1 slowly withdraw a measured quantity of gas (something less than 1 cc. so that when step 7 is taken the total gas can be read between the plunger and the meniscus). The vaseline plug will be seen to move down the tube as the gas is withdrawn.

6. Leave the cotton plug in place and pull the needle up until the point remains in the upper part of the vaseline. With a small hot spatula touch the outside of the test-tube opposite the track of the needle in the lower part of the vaseline plug. Leave the spatula in contact with the tube just long enough to close the opening in the vaseline.

7. Withdraw the needle from the tube, immediately stick the end into the dilute acid, and with the syringe held vertically draw up acid until the meniscus can be seen and read against the scale on the lower end of the syringe. Read the exact amount of gas (and air) in the syringe between the plunger and the meniscus of acid.

8. Stick the end of the needle into a 2 or 3 per cent solution of sodium hydroxide. With the apparatus in the position shown in Fig. 2 draw out the plunger until the alkali begins to enter the barrel of the syringe and neutralizes the acid there. As carbon dioxide is absorbed more alkali is automatically drawn into the syringe without further use of the plunger. With the end of the needle still in the alkali solution rock the syringe back and forth a number of times to insure complete absorption of the $CO_2$.

9. Again hold the syringe vertically with the plunger uppermost and read the amount of residual gas in the syringe. The difference between this reading and the one taken in step 7 equals the amount of $CO_2$ in the sample taken. The percentage of $CO_2$ in the sample is easily calculated and may be expressed as a gas ratio $\left(\frac{H_2}{CO_2}\right)$ if so desired.

We have tried a number of slight modifications of the details of the above technique but the above is probably the most perfect. The accuracy of the method is apparently limited only by the fineness of the graduations on the syringe. These should be at most 0.01 cc.

We are able to get almost as accurate results with 0.1 cc. of gas sample as with 1.0 cc. It is possible to determine the $CO_2$ content of a good sized bubble beneath the vaseline plug or in an agar shake culture. It is an advantage to select a tuberculin syringe having the following characteristics: (*a*) finely cut graduations, (*b*) a long slender barrel with considerable space above the graduations, (*c*) a well fitting plunger of colored glass with a sharp square-cut end which can be accurately read against the graduations on the colorless glass barrel. The carbon dioxide content of the small amount of atmospheric air contained in the needle and capillary rubber tube is a negligible quantity well within the limits of error. If the cultures are allowed to cool down to room temperature before the gas analysis is made and if the acid and alkali solutions are kept at the same temperature the temperature factor is also negligible. Formerly we took the precaution of allowing the end of the needle to be sealed by the vaseline (melted by the hot spatula) as it was withdrawn from the tube and then pricking this seal just as the needle was placed into the acid but it has been found unnecessary. There is no appreciable interchange of air and gas through the needle during the short time it is exposed to the air while being transferred from the culture tube to the dilute acid.

*Determination of Carbonates and $CO_2$ in the Medium.*

The carbonates and $CO_2$ in a fluid medium may be determined by means of the same apparatus. The equipment and technique are illustrated in Fig. 3. The steps of the technique are as follows:

1. Rinse the syringe and needle thoroughly with distilled water.
2. Dry and sterilize the needle in the flame.
3. With aseptic precautions place the needle so that it is held between the cotton plug and the side of the tube as for the determination of $CO_2$ above the medium.
4. Having placed the end of the syringe plunger at one of the graduation marks on the lower end of the barrel push the needle down the side of the tube through the vaseline plug (if vaseline is used) until the point is in the medium.
5. Draw the plunger of the syringe back just 0.1 cc.
6. Withdraw the needle with the same precautions as described in step 6 for the determination of $CO_2$ above the medium.
7. Draw into the needle about 0.03 cc. of air.

8. Stick the point of the needle into a small vial of capryl alcohol (colored with scarlet R) and draw 0.01 cc. into the needle, followed by 0.01 cc. of air.

9. Draw into the needle 0.05 cc. of 5 per cent sulfuric acid (colored with methyl red).

10. Holding the syringe vertically with the open needle uppermost draw back the plunger just until all of the red capryl alcohol and sulfuric acid are seen to be within the barrel of the syringe.

11. With the index finger of the left hand tightly stop up the end of the syringe where the rubber tube is attached and holding it as shown in Fig. 3 draw down the plunger while with a partial vacuum within the syringe the air leaks by the plunger and passes up through the contents of the syringe in a stream of fine bubbles. When the end of the plunger reaches a position a little beyond the graduations it should be allowed to remain here until the air ceases bubbling.

12. Turn the syringe to a vertical position with the plunger uppermost, release the finger from the rubber tube, push in the plunger to the beginning of the graduation marks, wait a few seconds to allow the fluid to drain down the sides of the syringe, and read the amount of gas and air present between the meniscus and the plunger.

13. Without changing the position of the syringe, needle hanging down, expel most of the fluid until the meniscus reaches the bottom of the syringe. Without admitting any air dip the end of the needle, now filled with fluid, into the sodium hydroxide solution and with the syringe held in the position shown in Fig. 2 draw up some of the alkali. Rock the syringe back and forth a few times. Again expel most of the fluid, holding the syringe in a vertical position and taking care to expel none of the gas. Draw in a fresh portion of sodium hydroxide and rock again.

14. Finally, holding the syringe vertically with needle hanging downward push the plunger in until its end reaches the graduation marks and read the amount of air remaining in the syringe. The difference between this reading and the one taken in step 12 equals the amount of $CO_2$ extracted from 0.1 cc. of medium, best expressed as volumes or as volumes per cent; *e.g.*, 0.1 cc. of $CO_2$ from 0.1 cc. of medium equals 1 volume or 100 volumes per cent.

It is our experience that more accurate results are obtained with samples of 0.1 cc. of medium than with larger amounts. This is doubtless due to the fact that in such a small syringe as we use the amount of air that can be passed through the sample and held within the syringe for measurement is limited and although sufficient for complete aeration of 0.1 cc. of medium is hardly sufficient for a larger sample. Without doubt larger samples could be employed in a longer syringe. It is an advantage to color the acid and alkali solutions with indicators, methyl red in the acid and thymol blue in the alkali, so that one may be sure of the reaction of the contents of the syringe at all times.

*Accuracy of the Determinations.*

The determinations in Table I were made from a single culture of an anaerobic organism in 5 cc. of plain bouillon under vaseline. The results illustrate the possibility of obtaining uniform results with multiple determinations and various methods of expressing the results.

TABLE I.

*Multiple Determinations of Carbon Dioxide Produced by an Anaerobic Organism in Plain Bouillon.*

Initial $CO_2$ content of medium = 0.0

Final hydrogen ion concentration of culture = pH 6.5

Total gas above medium at 37°C. = 1.05 volumes per 1.0 volume of medium.

| Determinations of $CO_2$ above medium. | | | | | | |
|---|---|---|---|---|---|---|
| Sample, *cc.* | 0.75 | 0.75 | 0.45 | 0.45 | 0.1 | 0.1 |
| $CO_2$, *per cent.* | 41.0 | 40.0 | 40.0 | 39.0 | 40.0 | 39.0 |
| $\frac{H_2}{CO_2}$ | $\frac{59}{41}$ | $\frac{60}{40}$ | $\frac{60}{40}$ | $\frac{61}{39}$ | $\frac{60}{40}$ | $\frac{61}{39}$ |
| $CO_2$ (vol. at 37°C.) | 0.43 | 0.42 | 0.42 | 0.41 | 0.42 | 0.41 |
| Determinations of $CO_2$ in medium* (samples = 0.1 cc.). | | | | | | |
| $CO_2$ (vol. at 20°C.) | 0.55 | 0.5 | 0.5 | 0.55 | 0.5 | 0.5 |

Computation of total $CO_2$.

Average; $CO_2$ above medium ..... 0.42 vol. at 37°C.

" $CO_2$ in medium .....0.52 vol. at 20°C. or 0.55 " " 37°"

Total $CO_2$ per 1.0 vol. of medium ..... 0.97 " " 37°"

* By $CO_2$ in medium is meant not only $CO_2$ present as such but also that present in the form of carbonates or carbonic acid.

To determine how much of the $CO_2$ present as carbonate might be recovered by the above method from water or from bouillon the following experiment was performed. 0.5066 gm. of sodium oxalate was converted into sodium carbonate by ignition in a platinum crucible. The sodium carbonate was dissolved in 10 cc. of distilled water and 3 cc. of this solution were diluted to 25 cc. with distilled water and with plain bouillon respectively for the determinations to be described.

As a control 3 cc. of distilled water were added to 22 cc. of the bouillon without addition of carbonate. Quadruple determinations were made of the $CO_2$ recovered from each of the three solutions. The amount of each sample taken was 0.1 cc. The results are presented in Table II.

TABLE II.

*Determinations of Carbon Dioxide in Standard Solutions of Sodium Carbonate in Water and in Bouillon.*

Room temperature = 20°C. Barometric pressure = 765.2 mm. Hg.
Hydrogen ion concentration of bouillon before addition of $Na_2CO_3$ = pH 7.3

| | Determinations. | | | | Average. |
|---|---|---|---|---|---|
| $CO_2$ from $Na_2CO_3$ in water..... | 1.05 | 1.05 | 1.05 | 1.03 | 1.045 vol. in 1.0 vol. of solution. |
| $CO_2$ " $Na_2CO_3$ " bouillon..... | 1.02 | 1.05 | 1.05 | 1.05 | 1.042 vol. in 1.0 vol. of solution. |
| $CO_2$ " $H_2O$ " " ..... | 0.02 | 0.0 | 0.03 | 0.0 | 0.012 vol. in 1.0 vol. of solution. |

Calculated theoretical volume of $CO_2$ in the above solutions of $Na_2CO_3$ in water and in bouillon = 1.066 volume in 1.0 volume of solution at 20°C. and 765.2 mm. Hg.

*Experiments Illustrating the Technical Possibilities of the Methods.*

Although the conditions under which a culture grows in the vaseline tube are not the same as those in the Smith fermentation tube and one would therefore not expect to obtain identical results in the two tubes, it seemed worth while to compare the results obtained by both methods with a view to interpreting their differences. A number of series of experiments have been performed with this end in view. The following is typical.

A strain of *Bacterium coli* was inoculated into six vaseline tubes and five fermentation tubes containing a certain lot of 2 per cent dextrose bouillon (pH = 7.2). By the 4th day of incubation all tubes had ceased to show changes in gas volume. The results of analysis of the gas above the media on the 4th day are recorded in Table III.

It should be noted that the total volume of gas produced above the medium in the two kinds of tubes cannot be compared because the

gas is measured in different terms. In the fermentation tube the gas collected comes from a diminishing amount of culture as gas formation forces the medium over into the open bulb and the gas is measured in terms of the capacity of the closed arm of the tube. In the vaseline tube the volume of the culture medium remains constant and the gas is measured in terms of volume of the medium. It is for this reason

TABLE III.

*Gas Formation by Dextrose Bouillon Cultures of Bacterium coli in Smith Fermentation Tubes and in Vaseline Tubes.*

| Fermentation tubes. | | Vaseline tubes. | |
|---|---|---|---|
| Gas above medium. | $\frac{H_2}{CO_2}$ | Gas above medium. | $\frac{H_2}{CO_2}$ |
| *per cent* | | *vol.* | |
| 65 | $\frac{53}{47}$ | 0.95 | $\frac{48}{52}$ |
| 41 | $\frac{55}{45}$ | 0.9 | $\frac{49}{51}$ |
| 46 | $\frac{55}{45}$ | 0.9 | $\frac{50}{50}$ |
| 49 | $\frac{51}{49}$ | 0.9 | $\frac{50}{50}$ |
| 51 | $\frac{56}{44}$ | 0.9 | $\frac{50}{50}$ |
| | | 0.9 | $\frac{50}{50}$ |

that we prefer to avoid the use of the term "per cent" in stating the volume of gas produced in the vaseline tube. It is difficult to see how one set of values can be translated into terms of the other. Aside from this fact it is noted that the results in the vaseline tubes are somewhat more uniform than those in the fermentation tubes. It is also found that invariably a larger proportion of $CO_2$ is formed under vaseline than in the closed arm of the fermentation tube. The reason for this is, as pointed out by Keyes (1909), that $CO_2$ is much more

soluble in water than is hydrogen and therefore not only does a considerable volume of $CO_2$ pass into solution in the medium but in the fermentation tube passes through the medium out into the air.

As a means of studying gas production the vaseline tube has many points of similarity to the long agar tube of Burri and Düggeli (1909) used by Frieber (1913, *a* and *b*) for gas analyses. In this tube, however, only solid medium was used and a layer of sterile non-nutrient agar was used instead of vaseline as a seal. The technical possibilities of the vaseline tube are much greater.

TABLE IV.

*Gas Formation by Dextrose Bouillon Cultures of Bacterium coli in Vaseline Tubes under Anaerobic and Aerobic Conditions.*

| Vaseline tubes (without air). | | Vaseline tubes (plus 1.0 vol. of air). | |
|---|---|---|---|
| Gas above medium. | $CO_2$ | Gas above medium. | $CO_2$ |
| *vol.* | *per cent* | *vol.* | *per cent* |
| 0.95 | 52 | 1.95 | 38 |
| 0.9 | 51 | 1.9 | 38 |
| 0.9 | 50 | 1.95 | 36 |
| 0.9 | 50 | 1.9 | 38 |
| 0.9 | 50 | 1.95 | 35 |
| 0.9 | 50 | 1.95 | 36 |
| Average.....0.91 | 50.5 | 1.93 | 36.8 |
| $0.91 \times 0.505 = 0.46$ vol. of $CO_2$ | | $1.93 \times 0.368 = 0.71$ vol. of $CO_2$ | |

In the methods of all the authors referred to above the cultures were grown under anaerobic conditions. Eldredge and Rogers (1914) and Osterhout (1918) have devised forms of apparatus for studying $CO_2$ production under aerobic or at least partially aerobic conditions. Some of the results obtained by the use of the tube of Eldredge and Rogers will be referred to later. The vaseline tube also permits the study of gas production under controlled aerobic conditions. After the medium in the tube has been inoculated and the vaseline allowed to solidify on the surface a measured volume of air or other gas may be injected beneath the vaseline plug by means of a syringe and the long needle used for gas analysis. The passage made by the needle in the vaseline is easily closed by means of a warm spatula as the needle

is withdrawn. In the experiment partially recorded in Table III was also included a series of six similar vaseline tubes of the same medium inoculated with the same strain of *Bacterium coli* but injected with an equal volume of air just after inoculation. Gas analyses were made at the same time as were those recorded in Table III. A comparison of the results with those of the vaseline tubes without air is given in Table IV.

Experiments were carried out with various volumes of air injected under the vaseline plug. With decreasing amounts of air below 1 volume the results gradually approach those obtained under anaerobic conditions. With 2 volumes of air the results were very nearly the same as with 1 volume. Naturally these proportions must be regarded as valid for this culture and medium only.

If from the 1.93 volumes of gas found in the tubes containing air the volume of air injected be subtracted, there remains 0.93 volume of gas produced by the culture, and since 0.71 volumes of $CO_2$ were present it might be computed that the gas ratio under aerobic conditions was $\frac{H_2}{CO_2} = \frac{22}{71}$, or $\frac{24}{76}$, as compared with $\frac{H_2}{CO_2} = \frac{50}{50}$ under anaerobic conditions. This calculation, however, assumes that the air injected is entirely inert and takes no part in the reaction. This we have reason to believe is not the case.

*Determination of Oxygen in the Gas above the Medium.*

The oxygen is readily determined in the same sample of gas used for the determination of $CO_2$ provided no atmospheric air is drawn into the syringe. This may be accomplished by having the space between the syringe plunger and the end of the needle filled with dilute acid instead of air as the needle is thrust through the vaseline plug. Otherwise the $CO_2$ is determined in the same manner described above and then the needle is dipped into a concentrated aqueous solution of pyrogallic acid. A little of the pyrogallic acid solution is drawn up into the syringe and as it mixes with the sodium hydroxide and absorbs the oxygen present more of the pyrogallic acid is automatically sucked up. The process is slower than the absorption of $CO_2$ by sodium hydroxide but, with constant rocking of the syringe back and forth, is complete in 2 or 3 minutes and the reading is made.

In another experiment with the same strain of *Bacterium coli* as used for the results given in Tables III and IV, both the $CO_2$ and the $O_2$ above the medium were determined and also the $CO_2$ in the medium. The results are given in Table V.

The outstanding feature of this experiment was the disappearance of the oxygen from the air above the aerobic culture. Assuming that

TABLE V.

*Further Gas Analysis of Dextrose Bouillon Cultures of Bacterium coli under Anaerobic and Aerobic Conditions.*

| | Vaseline tubes (without air). | | Vaseline tubes (plus 1.15 vol. of air). | |
|---|---|---|---|---|
| | Culture. | Control. | Culture. | Control. |
| | *vol.* | *vol.* | *vol.* | *vol.* |
| Gas above medium..... | 1.1 | 0.0 | 2.15 | 1.15 |
| $CO_2$ " " ..... | 0.5 | 0.0 | 0.75 | 0.04 |
| $CO_2$ in " ..... | 0.25 | 0.05 | 0.15 | 0.05 |
| Total $CO_2$..... | 0.75 | 0.05 | 0.9 | 0.09 |
| $O_2$ above medium..... | 0.0 | 0.0 | 0.0 | 0.07 |

Oxygen determined in atmosphere = 0.2 vol.

this was the case also in the experiment of Table IV, consideration of this factor would increase the gas ratio under aerobic conditions to $\frac{H_2}{CO_2} = \frac{37}{63}$ instead of $\frac{24}{76}$ since the space assumed to be occupied by oxygen was in reality occupied by some gas[2] other than $CO_2$ or $O_2$ produced by the culture. The disappearance of a part of the oxygen in the aerobic control tube noted in Table V was probably due to its passing into solution in the air-free medium which had been kept tightly sealed since it was autoclaved. The small amounts of $CO_2$ found in the controls may or may not be significant. It is not surprising that less $CO_2$ was found in the medium of the aerobic culture than in the anaerobic culture since the presence of air above the former favored the outward diffusion of $CO_2$. Taking all the figures into

[2] In stating the gas ratio $\left(\frac{H_2}{CO_2}\right)$ we have followed the custom of regarding all of the gas produced by the culture, other than $CO_2$, as hydrogen.

consideration it appears that *Bacterium coli* not only produces more $CO_2$ under aerobic conditions but a larger proportion of $CO_2$ with respect to $H_2$ or other gases.

Certain organisms which have been regarded as non-producers of gas have been found by special methods to produce appreciable amounts of $CO_2$. Such results are interesting and open up new possibilities for classification and for physiological study. However, they do not invalidate the practical value of formerly used methods for distinguishing between so called "gas-producing" and "non-gas-producing" organisms under stated conditions of cultivation. It was shown by Hesse (1893) that *Bacterium typhosus* and many other bacteria ordinarily considered to be non-gas producers do produce an appreciable amount of $CO_2$ and consume $O_2$ in the process. He calls this process the respiratory activity of bacteria.

Ayers, Rupp, and Mudge (1921) used the tube of Eldredge and Rogers (1914) to demonstrate $CO_2$ production by streptococci, and Nichols (1921) demonstrated $CO_2$ production by *Bacterium typhosus* by the same means. In this tube cultures are grown under aerobic conditions. Since, however, the atmosphere of the tube above the medium is kept free of $CO_2$ by the barium hydroxide solution we would expect to find less $CO_2$ in the medium than when the aerobic vaseline tube is used. To compare the results obtained in the vaseline tube with those of the Eldredge tube we must therefore consider the $CO_2$ in the medium as well as that above it in the vaseline tube. We have done this with two strains of streptococci for which we are indebted to Dr. Ayers. Strain X-4 was reported by Ayers, Rupp, and Mudge as producing $CO_2$ from dextrose and Strain 16H-1 was reported to produce $CO_2$ from Bacto-peptone. In Table VI are given the total $CO_2$ determinations of Ayers' strains of streptococci in various media. The determinations were made after incubation at 37°C. for 6 days. The amount of medium used was 3 cc. in each tube.

The resemblance between the $CO_2$ determinations of our aerobic cultures and those of Ayers, Rupp, and Mudge obtained by an entirely different method is so close that the differences seem insignificant. It is to be noted, however, that under anaerobic conditions much less $CO_2$ is produced in all media. Possibly this explains failure to demonstrate gas production by streptococci under the anaerobic conditions imposed by other methods.

Using the tube of Eldredge and Rogers, Nichols (1921) demonstrated $CO_2$ production by *Bacterium typhosus*. He obtained the maximum amount of $CO_2$ from cultures in 1 per cent glucose extract broth. In 2 per cent glucose veal infusion broth and with a different strain of typhoid bacillus, however, we have obtained even larger amounts of $CO_2$. The results of our determinations are recorded in Table VII.

TABLE VI.

*Determination of Total Carbon Dioxide Produced by Streptococci in Vaseline Tubes under Anaerobic and Aerobic Conditions.*

The medium was veal infusion bouillon containing 1 per cent Fairchild's peptone plus the substances indicated above each column.

| Strain. | Vaseline tubes (without air). | | | Vaseline tubes (plus 1.0 vol. of air). | | |
|---|---|---|---|---|---|---|
| | 3 per cent Fairchild's peptone. | 3 per cent Bacto-peptone. | 2 per cent dextrose. | 3 per cent Fairchild's peptone. | 3 per cent Bacto-peptone. | 2 per cent dextrose. |
| | *vol.* | *vol.* | *vol.* | *vol.* | *vol.* | *vol.* |
| Streptococcus X-4..... | 0.05 | 0.05 | 0.67 | 0.13 | 0.06 | 1.17 (1.25) |
| " 16H-1..... | 0.05 | 0.13 | 0.0 | 0.21 (0.12)* | 0.22 (0.33) | 0.13 (0.09) |

* The bold faced figures in parentheses are the results of Ayers, Rupp, and Mudge reduced to the same terms as ours. It should be noted, however, that whereas one of their media contained 4 per cent of Bacto-peptone ours used for comparison contained 3 per cent of Bacto-peptone and 1 per cent of Fairchild's peptone.

There was good growth of the typhoid bacillus under anaerobic conditions, though somewhat better under aerobic conditions. It is again seen that a really significant amount of $CO_2$ is produced under aerobic conditions only, and that a part but not all of the oxygen was consumed.

It must be pointed out that in interpreting the results of the determination of $CO_2$ in and above the medium certain very important factors must be taken into consideration. There is a very intimate relationship between temperature, hydrogen ion concentration of the culture, and the proportionate amount of $CO_2$ in and above the me-

dium. For instance, if the culture becomes alkaline, as may be the case with certain anaerobes, a large proportion of the $CO_2$ will be found in the medium as carbonates. If it becomes strongly acid a large proportion of the $CO_2$ will be found in the gas above the medium. If the culture is transferred from the incubator to the refrigerator the solubility of gases in the medium is appreciably increased. If the vaseline plug is broken so that atmospheric air gains access to the culture, $CO_2$ begins to pass out of the medium into the air. The latter can be demonstrated easily by breaking the seal of a culture and making periodic determinations of the $CO_2$ in the medium.

TABLE VII.

*Determination of Carbon Dioxide Produced by Bacterium typhosus in Dextrose Bouillon under Anaerobic and Aerobic Conditions.*

| | Vaseline tube (without air). | Vaseline tubes (plus 1.0 vol. of air). | |
|---|---|---|---|
| | Culture. | Culture. | Control. |
| | *vol.* | *vol.* | *vol.* |
| Gas above medium..... | 0.0 | 1.1 | 1.0 |
| $CO_2$ " " ..... | 0.0 | 0.14 | 0.03 |
| $CO_2$ in " ..... | 0.1 | 0.15 | |
| Total $CO_2$..... | 0.1 | 0.29 | |
| $O_2$ above medium..... | 0.0 | 0.06 | 0.13 |

Oxygen determined in atmosphere = 0.2 vol.

*Sterilization, Inoculation, and Cleaning of Vaseline Tubes.*

Before summarizing the advantages of the technique described it may be well to describe one or two simple points of technique which serve to make the vaseline tube almost as convenient to handle as an ordinary test-tube.

Autoclaving cannot be relied upon to sterilize vaseline since it is essentially a "dry" substance which the steam does not penetrate. It may be perfectly sterilized without visible alteration along with glassware in the hot air sterilizer at 175–185°C. for 2 hours. After being so sterilized it may be pipetted onto non-sterile medium in the tubes and then autoclaved with the medium. During sterilization air is driven from the medium and if the tubes are promptly cooled

after coming from the autoclave the medium under the solid vaseline plug is preserved free from air for long periods of time. There is no danger of spilling if tubes are turned over, and the medium may be kept at room or incubator temperature without evaporation. Since there is no water vapor in the tubes above the vaseline there is no tendency for molds to grow through the cotton plugs.

It is not necessary to pass a pipette through the vaseline to inoculate or add anything to the medium in the tube. In fact it may be inoculated with a platinum loop if the vaseline seal is opened as shown in Figs. 4 and 5. The vaseline is melted by directing the flame of a micro burner against the side of the tube. The tube is then slanted in a dish or tray of water as shown in Fig. 4. After the vaseline has hardened as a layer over the slanted surface of the medium the tube is rotated and gently tapped by the fingers, as shown in Fig. 5, until the vaseline over the medium flaps up and adheres to the side of the tube, exposing the medium for inoculation. The seal is again closed by melting the vaseline by means of the flame directed against the outside of the tube in the region of the vaseline. The medium need not be perceptibly heated by the process and with reasonable care tubes rarely crack.

Tubes of discarded cultures are cleaned as follows: The tubes are placed upright in a basket and autoclaved. While they are still hot the cotton plugs are withdrawn, the basket is placed in a deep pail or other vessel somewhat deeper than the tubes, and the tubes, remaining upright, are filled and covered with hot water. The pail of water is heated on a burner while the vaseline rises to the surface whence after cooling it may be recovered if desired. The tubes may then be washed by the usual method.

## SUMMARY.

There has been described the use of the vaseline tube and the tuberculin syringe for the study of gas production by bacteria.

A comparison is made of some of the results obtained by the use of the method here described, the Smith fermentation tube, and the tube of Eldredge and Rogers.

The reports of $CO_2$ production by certain streptococci by Ayers, Rupp, and Mudge and by *Bacterium typhosus* by Nichols have been confirmed by the author's method.

The data presented serve to illustrate the accuracy and technical possibilities of the method.

In addition to economy of glassware, medium, and labor, the vaseline tube and syringe method of micro gas analysis possesses the following advantages. (1) Gas produced above either liquid or solid media may be measured and analyzed. (2) The gas produced may be measured in terms of a definite and constant quantity of medium used. (3) The vaseline tube provides a closed system from which gases do not escape into the air. (4) Separate determinations of the $CO_2$ produced in and above fluid media may be made. (5) Determinations may be made from very small samples of material. (6) Numerous gas analyses of the same culture may be made at various times during the growth of the culture without contaminating or destroying it. (7) Gas production may be observed under both anaerobic and controlled aerobic conditions.

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## EXPLANATION OF PLATES.

### PLATE 55.

FIG. 1. Withdrawing the sample of gas from beneath the vaseline plug.

### PLATE 56.

FIG. 2. Sodium hydroxide solution being drawn into the syringe for the absorption of carbon dioxide.

FIG. 3. The aeration of a sample of culture for the determination of the carbon dioxide in the medium.

### PLATE 57.

FIG. 4. The tube of medium slanted but still covered by a layer of vaseline.

FIG. 5. The slanted tube rotated causing the lower end of the vaseline seal to flap up, thus exposing the medium for inoculation.

FIG. 1.

(Brown: Micro gas analysis of bacterial cultures.)

FIG. 2.

FIG. 3.

(Brown: Micro gas analysis of bacterial cultures.)

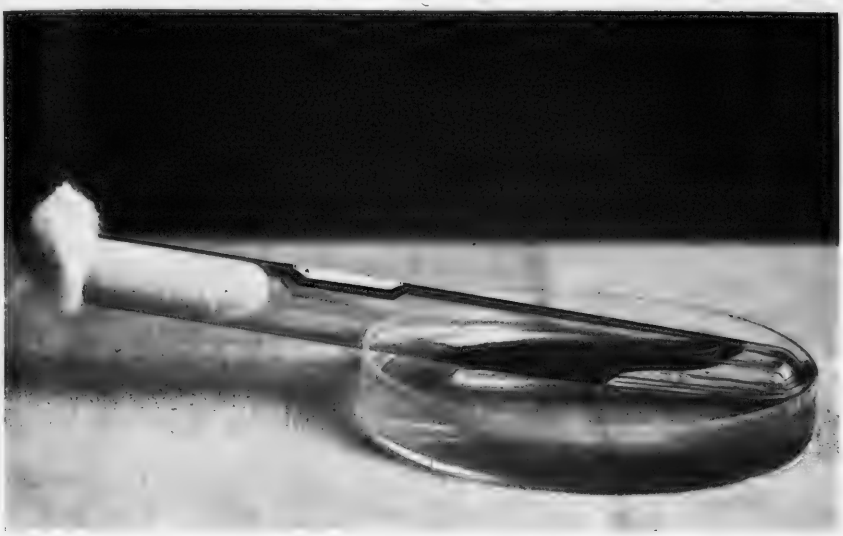

FIG. 4.

FIG. 5.

(Brown: Micro gas analysis of bacterial cultures.)

# STUDIES ON THE PNEUMOCOCCUS.

## I. Acid Death-Point of the Pneumococcus.

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(Received for publication, December 16, 1921.)

In a previous publication[1] we brought forward evidence indicating that in the growth and death of the pneumococcus in fluid cultures containing 1 per cent glucose the production of acid was the most important bactericidal factor and that such cultures when allowed to grow and die out usually reach a final pH of about 5.1. In our experiments on the relation of the time of exposure to the death of the organisms we made use of weak solutions of bouillon at varying hydrogen ion concentrations. In order to exclude the possibility that an unsuitable medium might play a part in these results, we have since used a stronger broth adjusted to approximate isotonicity, as shown in Table I.

5 cc. from each flask (Table I) were removed to sterile test-tubes and each tube of the series was inoculated with 2 drops of a saline suspension of washed living pneumococci. Transplants were made on blood serum after varying periods of incubation. Table II indicates the results obtained in an experiment with Type I pneumococcus. No noteworthy change in the hydrogen ion concentration of the tubes occurs during the experiment. It will be seen that at a pH of 4.47 no living organisms were obtained after an interval of 1 hour. At a pH of 5.15 living organisms were obtained after 1 hour but not after 2. At a pH of 5.78 organisms were living after 2 hours but not after 3, while at a pH of 6.33 organisms were obtained after 6 hours. At a pH of 6.63 to 7.80 inclusive transplants were positive from 8 to 10 days.

A considerable number of similar experiments has been performed

[1] Lord, F. T., and Nyc, R. N., *J. Exp. Med.*, 1919, xxx, 389.

TABLE I.

*Isotonic Broth for Testing the Acid Death-Point of the Pneumococcus.*

| 125 cc. of double strength bouillon (broth made with double strength beef infusion) hydrochloric acid, 0.5 M phosphates, and water to 237.5 cc. | | | | | | | | 190 cc. from each flask and sodium chloride solution to depress freezing point, plus water to approximately 200 cc. | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Flask No. | pH* | 0.5 M $KH_2PO_4$ | $Na_2HPO_4$ | HCl | | NaOH (concentrated). | Depression of freezing point. | 10 per cent NaCl. | $H_2O$ | Final depression of freezing point. | Final pH.† |
| | | | | $\frac{N}{1}$ | Concentrated. | | | | | | |
| | | cc. | cc. | cc. | cc. | cc. | °C. | cc. | cc. | °C. | |
| 1 | 4.9 | 15.0 | | 1 | 0.3 | | 0.450 | 6.7 | 3.3 | 0.620 | 4.47‡ |
| 2 | 5.3 | 15.0 | | 1 | 0.2 | | 0.378 | 7.4 | 2.6 | 0.622 | 5.15 |
| 3 | 5.75 | 15.0 | | 1 | 0.1 | | 0.390 | 8.0 | 2.0 | 0.584 | 5.78 |
| 4 | 6.1 | 15.0 | | | | | 0.322 | 11.4 | | 0.638 | 6.33 |
| 5 | 6.4 | 13.8 | 1.2 | | | 0.3 | 0.378 | 8.4 | 1.6 | 0.598 | 6.63 |
| 6 | 6.9 | 9.45 | 5.55 | | | 0.3 | 0.349 | 9.5 | 0.5 | 0.571 | 7.01 |
| 7 | 7.5 | | 15.00 | | | | 0.328 | 11.0 | | 0.700 | 7.51 |
| 8 | 7.8 | 1.2 | 13.8 | | | 0.1 | 0.305 | 11.0 | | 0.621 | 7.80 |

* Determined colorimetrically.

† Determined electrometrically.

‡ The figure in the hundreds place is uncertain by this method.

TABLE II.

*Acid Death-Point of the Pneumococcus. Time Relation at Varying Hydrogen Ion Concentrations.*

| Flask No. | pH* | Results obtained on transplants after varying intervals. | | | | | | | | | | | | | | | | | | Final pH.† |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 hr. | 2 hrs. | 3 hrs. | 4 hrs. | 5 hrs. | 6 hrs. | 7 hrs. | 24 hrs. | 48 hrs. | 72 hrs. | 4 days. | 5 days. | 6 days. | 7 days. | 8 days. | 9 days. | 10 days. | 12 days. | 13 days. | |
| 1 | 4.47 | 0‡ | 0 | | | | | | | | | | | | | | | | | | 4.47 |
| 2 | 5.15 | + | 0 | 0 | | | | | | | | | | | | | | | | | 5.15 |
| 3 | 5.78 | + | + | 0 | 0 | | | | | | | | | | | | | | | | 5.72 |
| 4 | 6.33 | + | + | + | + | + | + | 0 | 0 | | | | | | | | | | | | 6.19 |
| 5 | 6.63 | + | + | + | + | + | + | + | + | 0 | + | + | + | + | + | + | 0 | 0 | | | 6.58 |
| 6 | 7.01 | + | + | + | + | + | + | + | + | + | + | 0 | + | + | + | + | 0 | 0 | | | 6.58 |
| 7 | 7.51 | + | + | + | + | + | + | + | + | + | + | + | + | + | 0 | + | 0 | 0 | | | 7.14 |
| 8 | 7.80 | + | + | + | + | + | + | + | + | + | 0 | + | + | + | + | 0 | + | + | 0 | 0 | 7.30 |

* Determined electrometrically.

† Determined colorimetrically.

‡ 0 indicates lack of growth on the transplant; +, growth.

with Type I and II pneumococci. Though there is slight variation in the time element in the bactericidal action of acidity, the results are essentially the same for the two types and confirm the results previously obtained and reported; at a pH of about 5.1 or lower the pneumococcus does not survive longer than a few hours, at a pH of about 6.8 to 7.4 the pneumococcus may live for at least many days, and in the intervening solutions, between 6.8 and 5.1, the organism is usually killed with a rapidity which bears a direct relation to the hydrogen ion concentration; *i.e.*, the greater the acidity the more rapid the death.

For technical assistance in the experiments in this and the subsequent articles in this series we are indebted to Miss Ruth Seybolt and Miss Elnora Reed Blanchard.

# STUDIES ON THE PNEUMOCOCCUS.

## II. Dissolution of Pneumococci at Varying Hydrogen Ion Concentrations. Effect of Temperature, Previous Killing of the Organisms, and Fresh Human Serum on the Phenomenon. Behavior of Other Organisms.

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In a previous publication[1] we have called attention to the dissolution after incubation of living washed pneumococci, suspended in standard solutions of known hydrogen ion concentration. There is clearing of the suspensions from disintegration of the organisms within the range of pH of about 5.0 to 6.0 within an hour, and diminishing density with longer incubation in the more alkaline side of the scale. In discussing the phenomenon, we suggested that an enzyme derived from the organisms might be responsible for their dissolution. Further experiments have shown that the phenomenon is constantly observed with all the strains of pneumococci tested, including examples of the types commonly known as Types I, II, and III.

Avery and Cullen[2] have since demonstrated the presence in the pneumococcus of an erepsin-like enzyme, a lipase, an invertase, an amylase, and an inulase, with an activity within a zone of hydrogen ion concentration which bears a striking correlation to that of the biologic activity of the bacterial cell.

To obtain further information on the dissolution phenomenon, it seemed desirable (1) to suspend the organisms in another solution than the standard, (2) to determine the effect of temperature on the phenomenon, (3) to observe the behavior of dead as well as living pneumococci, (4) to test the effect of fresh human serum on the dissolution of pneumococci, (5) to compare the rate of dissolution of pneumococci

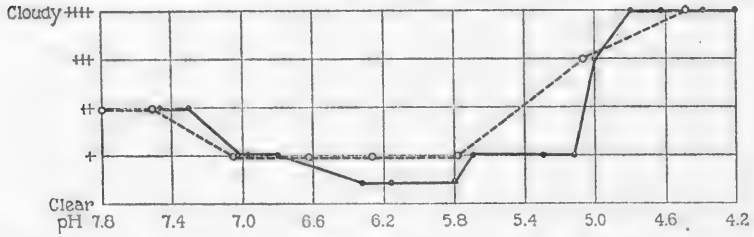
[1] Lord, F. T., and Nye, R. N., *J. Exp. Med.*, 1919, xxx, 389.

[2] Avery, O. T., and Cullen, G. E., *J. Am. Med. Assn.*, 1920, lxxiv, 1668; *J. Exp. Med.*, 1920, xxxii, 547, 571, 583.

in a fresh standard solution at pH 6.1 with that in a standard solution of the same pH in which the organisms had previously undergone dissolution, and (6) to do comparative dissolution tests on pneumococci and other organisms.

*1. Dissolution of Pneumococci in Nutrient Broth at Varying Hydrogen Ion Concentrations. Comparison with Dissolution in Standard Solutions.*—To observe the effect of suspending pneumococci in a medium in which the conditions are suitable for growth and multiplication at the proper range of pH, a double strength meat infusion broth was made and the pH and tonicity were adjusted as shown in the preceding article.[3]

To each of eight tubes containing 0.2 cc. of approximately isotonic ($\Delta = 0.580 - 0.748$°C.) standard solution at varying hydrogen ion

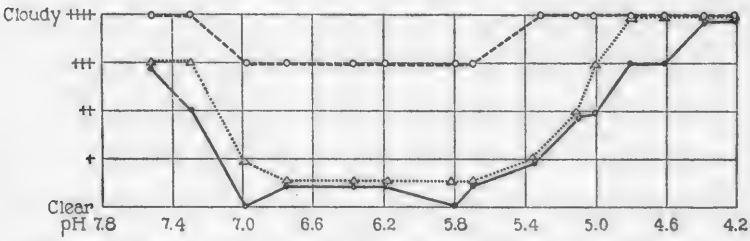



TEXT-FIG. 1. Dissolution of pneumococci in nutrient broth and standard solutions at varying hydrogen ion concentrations. Comparison of the dissolution of Pneumococcus Type I at the end of 24 hours at varying hydrogen ion concentrations in isotonic bouillon (broken line) and in isotonic standard solutions (solid line).

concentrations and to each of a similar number of tubes containing an equal amount of approximately isotonic nutrient broth at varying hydrogen ion concentrations, 0.2 cc. of a saline suspension of living washed Pneumococci Type I was added. The two series were incubated. No change in the suspensions was observed at the end of 1 hour. At the end of 6 hours there was slight dissolution in both series at about the same range of pH; *i.e.*, at 5.78 to 7.8 in the bouillon series and 5.14 to 6.98 in the standard solutions. The result at the end of 24 hours is shown in Text-fig. 1. The dissolution in the two series was practically the same and almost complete at a pH of about 5.7 to 7.0.

[3] Lord, F. T., and Nye, R. N., *J. Exp. Med.*, 1922, xxxv, 686, Table I.

*2. Effect of Temperature on Dissolution of Pneumococci at Varying Hydrogen Ion Concentrations.*—Living washed Pneumococci Type I were suspended in approximately isotonic standard pH solutions at incubator and room temperature (23.2°C.) and in the ice box (12.2°C.). The series used for the test in the ice box was placed on ice and when thoroughly chilled a chilled suspension of organisms was added. The tubes were then replaced on ice. Observations were made after 1, 6, and 24 hours. No change in the density of the suspensions was noted after 1 hour. After 6 hours no change was observed in the suspensions at room and ice box temperature, but in the suspensions at incubator temperature there was slight clearing at pH 4.81 and 5.02, more marked clearing at pH 5.14 to 6.98, and slight clearing at pH 7.3 to 7.49. The appearance of the tubes after 24 hours is indicated in

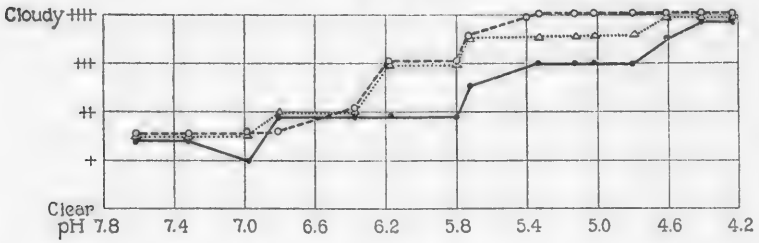



TEXT-FIG. 2. Effect of temperature on the dissolution of pneumococci. Comparison of the degree of dissolution of living Pneumococcus Type I suspended in isotonic standard pH solutions in the incubator (solid line), at room temperature (dotted line), and in the ice box (broken line). The observations were made after 24 hours.

Text-fig. 2. At the expiration of this interval dissolution had proceeded to about the same degree at incubator and room temperature and was almost complete at pH 5.33 to 6.98. Though dissolution occurs after 24 hours at ice box temperature and at the same range of pH, it is much less marked than at incubator and room temperature. After the expiration of 48 hours, however, the suspensions at ice box temperature had reached the same degree of dissolution as those at incubator and room temperature from a pH of 5.8 to 6.98 inclusive. Dissolution in the ice box was somewhat less at pH 5.02 to 5.72 inclusive.

Dissolution therefore takes place at room and ice box temperature, though much more slowly than at incubator temperature. It may be noted in this as in the preceding experiment that dissolution occurs more quickly at a certain critical range; *i.e.*, from about pH 5.14 to 6.98.

*3. Effect of Previous Death of Pneumococci on the Dissolution Phenomenon.* (*a*) *Dissolution of Pneumococci Allowed to Grow and Die Out.*—A glucose broth culture of pneumococci was allowed to grow and die out. At the end of about 72 hours the pH was 5.02 and a transplant was sterile. The culture was centrifuged, the sediment suspended in isotonic ($\Delta = 0.62°$C.) saline solution, again centrifuged, and resuspended in isotonic saline solution. An equal amount (0.2 cc.) of suspended organisms was added to three sets of approxi-

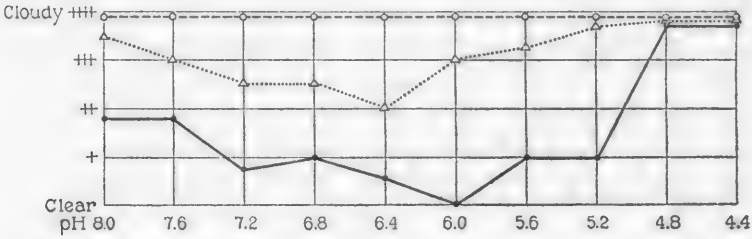



TEXT-FIG. 3. Effect of previous death of pneumococci on dissolution. Comparison of the degree of dissolution of Pneumococcus Type I allowed to grow and die out in glucose broth, suspended in isotonic standard solutions, at incubator temperature (solid line), at room temperature (dotted line), and in the ice box (broken line). The observations were made after 24 hours.

mately isotonic standard pH solutions, ranging from 7.49 to 4.23. One series was placed in the incubator, another in the ice box (11.2°C.), and the third left at room temperature (20.0°C.). Observations were made after 1, 18, and 24 hours. Dissolution slowly proceeded to about the same degree in all three sets and at about the same range of pH. The results are shown in Text-fig. 3. The degree of dissolution of dead organisms increases progressively from the acid toward the alkaline end of the scale and is in general most complete at a pH of 7.0 to 7.49. On comparison of the dissolution of living (Text-fig. 2) and dead organisms (Text-fig. 3) a difference is seen in the curves. After 24 hours the dissolution of living organisms is most marked and

almost complete within a range of pH from 5.72 to 6.98, and takes place little or not at all at the more alkaline and more acid ends of the scale respectively. Death of pneumococci allowed to grow and die out may be ascribed to acid production in the media, and the dissolution curve of organisms thus killed is different from that of organisms killed by heat (*cf.* Text-fig. 4).

(*b*) *Dissolution of Pneumococci Killed by Heat.*—To determine the effect of previous killing of the pneumococci by heat, one lot of organisms at the height of their growth in dextrose bouillon was heated to 100°C. for 5 minutes, a second lot was heated to 57°C. for 1 hour, and a third lot of living organisms was reserved as a control. Transplants from the flasks heated at 100°C. for 5 minutes and 57°C. for 1 hour were sterile. All three lots were centrifuged, washed with




TEXT-FIG. 4. Effect of heating pneumococci on dissolution. Comparison of the degree of dissolution of Pneumococcus Type II, living (solid line), killed by heat at 57°C. for 1 hour (dotted line), and killed by heat at 100°C. for 5 minutes (broken line). The observations were made after 24 hours in the incubator.

sterile isotonic saline solution, and recentrifuged. The supernatant saline solution was pipetted off and a cloudy suspension of the organisms made by adding fresh sterile isotonic saline solution, care being taken to secure suspensions of equal density in the three groups. The three lots were then mixed with standard pH solutions, placed in the incubator, and the results noted at intervals. As shown in Text-fig. 4, after 24 hours the suspensions of living pneumococci showed the usual dissolution curve, the clearing of the suspension being most marked at a pH of 6.0. No dissolution was noted at a pH of 4.8 and 4.4. Considerable, though less dissolution than at a pH of 6.0 was observed in the intervening acid end of the scale and a gradually dimin-

ishing clearing of the tubes from a pH of 6.0 toward the more alkaline end of the scale. Some dissolution of pneumococci killed by heating at 57°C. for 1 hour was observed, the general character of the curve being similar to that with the living organisms. Previous heating of the organisms at this temperature had evidently diminished their susceptibility to dissolution at the critical hydrogen ion concentrations. The pneumococci heated to 100°C. for 5 minutes showed no change in the density of the different tubes.

*4. Effect of Fresh Human Serum on Dissolution of Pneumococci at Varying Hydrogen Ion Concentrations.*—In view of the well established presence of antienzyme in normal serum it seemed desirable to determine its effect on the dissolution of pneumococci. For this purpose 0.5 cc. of fresh human serum was placed in each of seven sterile test-tubes. To each tube was added 2 drops of buffer solution containing equal parts of 0.5 M $KH_2PO_4$ and 0.5 M $Na_2HPO_4$. 2 drops of indicator were also added to each tube, 1 per cent aqueous sodium alizarin sulfonate being used at pH 3.6 to 5.6 inclusive and 0.01 per cent aqueous phenolsulfonephthalein at pH 6.1 to 7.8 inclusive.[4] The fluid in each tube was brought to the desired pH by the addition of drops of N HCl, 0.2 N HCl, N NaOH, or 0.2 N NaOH. The number of drops added to each tube was recorded and those receiving less than the maximum number (8 drops) were made up to volume with sterile saline solution. The comparator rack method was used in the construction of the series. Sterile precautions were used throughout and cultures from the completed series were negative.

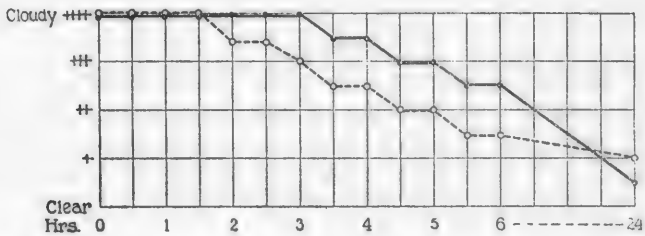
To each tube in the two series 0.2 cc. of a heavy suspension of washed living Pneumococci Type I was added. The two sets were incubated for 36 hours. The pH did not change during this interval. Macroscopic changes in density were difficult of appreciation and reliance was placed on microscopic examination of stained smears. After 36 hours, examination of stained smears from the tubes containing organisms suspended in serum at varying pH showed no apparent dissolution in any of the tubes. The organisms suspended in standard pH solutions showed the usual curve of dissolution.

Suspension of pneumococci in normal serum at varying hydrogen ion

[4] Experiments have shown that these two indicators themselves do not prevent dissolution and the inhibition is to be ascribed to the serum.

concentrations therefore prevents the dissolution of the organisms at the critical range of pH.

*5. Comparison of the Degree of Dissolution of Pneumococci in Standard Solution of pH 6.19 with Dissolution in the Supernatant Fluid of the Standard Solution of the Same pH in Which Large Numbers of Pneumococci Have Been Previously Dissolved.*—The most likely explanation of the dissolution of pneumococci at the critical range of pH in standard solutions seems to be the activation of an intracellular enzyme. If the enzyme is set free by the process of dissolution and exists in the fluid in which the organisms are undergoing disintegration, then dissolution of a fresh lot of pneumococci should take place at a more rapid rate in fluid in which dissolution has already taken place than




TEXT-FIG. 5. Comparison of the rapidity of dissolution of Pneumococcus Type I at pH 6.19 in the supernatant fluid of standard solution in which pneumococci have been previously dissolved (broken line), and in fresh standard solution (solid line).

in fresh standard solutions at the same pH. For the purpose of testing this hypothesis the following experiment was performed.

The contents of a liter flask of glucose bouillon culture of living Pneumococci Type I were centrifuged. The sediment was collected, washed in sterile isotonic ($\Delta = 0.620°$C.) saline solution, recentrifuged, and suspended in 2 cc. of isotonic salt solution. This suspension of living pneumococci was added to 5 cc. of isotonic standard solution of pH 6.15 and incubated for 48 hours. At the end of this time the suspension was centrifuged. In one test-tube was placed a part of the supernatant fluid thus obtained and in another an equal amount of fresh standard solution of pH 6.19. Equal amounts of a heavy suspension of living Pneumococci Type I were now added to both tubes and the result

was noted after incubation[5] (Text-fig. 5). At the expiration of ½ hour no difference was observed in the density of the two suspensions. After 2 hours the suspension of organisms in the supernatant fluid had cleared to a perceptibly greater degree than that in the fresh standard solution. Further observation over a period of 6 hours continued to show greater dissolution in the supernatant fluid. At the expiration of 24 hours both tubes had cleared, a result to be expected in suspensions of pneumococci at pH 6.19 after a sufficient interval. Observations were made by gross and microscopic examination. The pH did not change during the experiment. Repeated experiments showed the same result.

The theory was therefore confirmed that dissolution of pneumococci takes place more rapidly in standard solution of pH 6.19 in which large numbers of organisms have been previously dissolved than in fresh standard solution at the same pH.

*6. Dissolution Tests with Other Organisms at Varying Hydrogen Ion Concentrations.*—Tests with *Streptococcus viridans* and *hæmolyticus* and *Staphylococcus aureus*, similarly performed with washed suspensions of living organisms in standard solutions at incubator temperature, showed no dissolution.

## DISCUSSION.

The phenomenon of dissolution of pneumococci at critical hydrogen ion concentrations seems to bear a definite relation to the living organisms. It is most marked when living organisms are used in the experiment and takes place most quickly at a range of hydrogen ion concentrations which is slightly less acid (pH 5.5 to 6.7) than the limiting hydrogen ion concentration (pH 5.1) of growing glucose bouillon cultures and slightly more acid than the most acid reaction (pH 6.8) at which growth of organisms can be continued. It occurs, however, with the lapse of time throughout the whole range of hydrogen ion concentrations (pH 6.8 to 7.8) at which the pneumococci are biologically active.

[5] Both at the beginning and end of the experiment the suspensions of pneumococci in the supernatant fluid are somewhat more cloudy than the suspensions in fresh standard solution because of the presence of fine particles of pneumococci not removed by the centrifuge.

As we have already noted in our previous paper, the most probable explanation seems to be the activation of an enzyme derived from the bacteria themselves. Avery and Cullen's[2] demonstration of the presence of enzyme in bile and in phosphate solutions of pH 6.2 in which pneumococci have undergone disintegration lends support to this point of view. The fact that the phenomenon is most marked with living organisms at incubator temperature, the inhibiting effect of death of the organisms (Text-figs. 3 and 4) and of the addition of fresh human serum to the solutions, and the greater rapidity with which dissolution takes place in standard solutions in which pneumococci have been previously dissolved (Text-fig. 5) are consistent with enzymatic action. If merely the effect of acidity on ferment action is considered, the absence of dissolution at acidities greater than about pH 4.6 coincides closely with the failure of Avery and Cullen to demonstrate any appreciable ferment activity at acidities greater than pH 4.5. The less complete dissolution and change in the character of the curve of dissolution of pneumococci allowed to grow and die out in glucose bouillon (Text-fig. 3) may be due to partial destruction of the enzyme or a change in the physical state of the bacterial cell in consequence of exposure to this acidity (pH 5.02). Avery and Cullen have shown that the activity of the peptonase and lipase obtained from the pneumococcus is not appreciably diminished by exposure to pH 5.0 for 2 hours. It may be, however, at this hydrogen ion concentration, which corresponds closely with the isoelectric points and coincident precipitation optima of various biologically important proteins, that some change has occurred to diminish the permeability of the cell membrane or to change the physical state of the bacterial cell protoplasm. Less dissolution when the pneumococci are killed by heat and the absence of dissolution after heating to 100°C. may be ascribed to partial and complete destruction respectively of the enzyme at these temperatures.

Assuming that dissolution occurs in consequence of the death of the organisms and the activities of an endocellular enzyme, the disintegration may be conceived to take place most actively at that point in the scale where the largest number of organisms are most rapidly killed with, at the same time, a minimum of injury to the cell membrane which still remains permeable for the hydrogen ions and permits the activation of the endobacterial ferment.

Enzymatic action at ice box temperature is at variance with the usual temperature range of ferment action. Dissolution proceeded much more slowly at this temperature but was finally as complete as at incubator temperature.

## CONCLUSIONS.

Suspensions of living pneumococci in approximately isotonic standard solutions and in approximately isotonic bouillon with pH varying from about 4.0 to 8.0 after incubation show dissolution of organisms in those solutions having a pH higher than about 5.0. Dissolution is most marked at a critical range of about pH 5.0 to 7.0. Some dissolution also takes place toward the more alkaline end of the scale. No dissolution occurs at the most acid end of the scale.

Dissolution in the standard solutions occurs at incubator, room, and ice box temperature. It is less marked at ice box temperature. Dissolution takes place in standard pH solutions with pneumococci allowed to grow and die out in glucose bouillon but unlike dissolution with living organisms is progressive from the acid toward the alkaline end of the scale. Pneumococci killed by heat for 1 hour undergo less dissolution than living organisms, the general character of the curve being similar to that with living organisms. Pneumococci killed by heat at 100°C. for 5 minutes do not undergo dissolution. The addition of fresh human serum to the suspensions of pneumococci at varying pH prevents dissolution. Dissolution of pneumococci takes place more rapidly at pH 6.1 in standard solutions in which large numbers of pneumococci have been previously dissolved than in fresh standard solutions at the same pH.

The dissolution of pneumococci under the conditions of the experiments may be ascribed to an enzyme derived from the bacteria themselves.

Other organisms such as *Streptococcus viridans* and *hæmolyticus* and *Staphylococcus aureus* do not undergo dissolution under conditions similar to those to which the pneumococcus was exposed.

# STUDIES ON THE PNEUMOCOCCUS.

## III. Dissolution of Pneumococci in Pneumonic Cellular Material at Varying Hydrogen Ion Concentrations. Resistance of Certain Other Organisms to Dissolution.

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To determine the effect at varying hydrogen ion concentrations of cellular material obtained from the pneumonic lung on pneumococci and other organisms, the following experiments were performed.

The mash from a pneumonic lung due to Type I pneumococcus and preserved with chloroform and toluene was ground in a sterile mortar with some of the saline solution with which the material was originally put up. The resulting cellular material was allowed to stand on ice until the supernatant fluid was clear. The supernatant fluid and sediment were put on Löffler's blood serum. Both produced proteolysis, the cells more than the supernatant fluid. The pH of this fluid was 5.5.

The pH of the cellular material was changed by adding to 10 cc. 9 drops of normal NaOH, which gave a pH of 6.95. To another 10 cc. lot 1 drop of concentrated HCl was added, with a pH of 4.5 as the result.

*Dissolution Experiment with Pneumococci.*—The cellular material of varying hydrogen ion concentrations thus prepared was set up with suspensions of Type I, II, and III pneumococci as shown in Table I. As will be seen from the table the behavior in the cellular suspension at varying hydrogen concentrations was practically the same for the three types. All three types at a pH of 4.5, after incubation over night, for the most part retain their morphology. Type I pneumococcus at this pH remained Gram-positive while Types II and III became Gram-negative. All three types underwent dissolution at a pH of 5.5 and 6.95.

The dissolution of pneumococci taking place in cellular material from the pneumonic lung at a pH of 6.95 and 5.5 may be ascribed to the action of an enzyme operative at this pH. Previous experiments indicate that the enzyme may be derived from the bacteria themselves, since similar dissolution of the organisms at this pH is noted in stand-

TABLE I.

*Relation of Hydrogen Ion Concentration to Dissolution of Pneumococci in Cellular Material from the Pneumonic Lung.*

| Tube No. | Amount of cellular suspension. | | | Amount of pneumococcus suspension. | | | Smears. | |
|---|---|---|---|---|---|---|---|---|
| | pH 4.5 | pH 5.5 | pH 6.95 | Type I. | Type II. | Type III. | Made at once. | Made after incubation over night. |
| | *gtt.* | *gtt.* | *gtt.* | *gtt.* | *gtt.* | *gtt.* | | |
| 1 | 5 | | | 5 | | | Organisms numerous, in good condition, and all Gram-positive. | Organisms well preserved, in good condition, and Gram-positive. |
| 2 | | 5 | | 5 | | | | Organisms have disintegrated and disappeared. |
| 3 | | | 5 | 5 | | | | Organisms have disintegrated and disappeared. |
| 4 | 5 | | | | 5 | | | Organisms well preserved but all Gram-negative. |
| 5 | | 5 | | | 5 | | | Organisms have disintegrated and disappeared. |
| 6 | | | 5 | | 5 | | | Organisms have disintegrated and disappeared. |
| 7 | 5 | | | | | 5 | | Organisms mostly well preserved, a few slightly eroded; all Gram-negative. |
| 8 | | 5 | | | | 5 | | Organisms mostly disintegrated; an occasional Gram-negative organism seen. |
| 9 | | | 5 | | | 5 | | Organisms have disintegrated and disappeared. |

ard solutions or bouillon without cellular material. An enzyme derived from the cellular material may also, however, be a factor in the dissolution.

*Dissolution Experiment with Other Organisms.*—To a cellular suspension from a Type I pneumonic lung with a pH of 5.5, suspensions

of living and washed *Streptococcus hæmolyticus* and of *Streptococcus viridans* were added. To another portion of the same cellular suspension, Type I pneumococcus was also added as a control. In setting up the experiment 5 drops of the cellular suspension were added to 5 drops of a suspension of the different organisms. Microscopic examination of the mixtures after incubation over night showed that the pneumococci had disappeared with the exception of an occasional Gram-positive organism. There were many fragments of Gram-positive organisms and many staining points. *Streptococcus hæmolyticus* and *Streptococcus viridans* were not dissolved and remained Gram-positive after exposure in the incubator to the cellular suspension.

## CONCLUSIONS.

Pneumococci of Types I, II, and III undergo dissolution when mixed with cellular material from the pneumonic lung at a pH of 6.95 and 5.5, but not at a pH of 4.5. An enzyme derived from the bacteria themselves or from the cellular material may be the cause of the dissolution. *Streptococcus hæmolyticus* and *Streptococcus viridans* do not undergo dissolution under similar experimental conditions.

# STUDIES ON THE PNEUMOCOCCUS.

## IV. Effect of Bile at Varying Hydrogen Ion Concentrations on Dissolution of Pneumococci.

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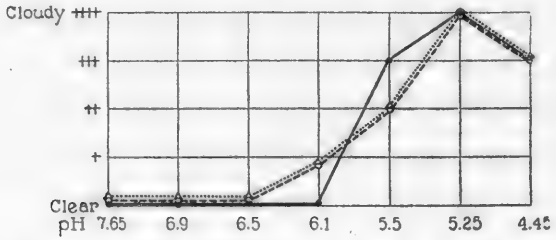
Fresh beef bile, obtained from the slaughter house, after sterilization for 15 minutes in the autoclave, was found to have a pH of 7.8. By the addition of increasing amounts of HCl to different lots of this bile a series was made with a pH range of 7.65 to 4.45 as determined by the dialysis method (Table I). 4 drops from each of the series of tubes containing bile at varying pH were removed to four sets of seven test-tubes. To one set were added 4 drops of a suspension of Pneumococcus Type I, to a second an equal amount of a suspension of Type II, and to a third a suspension of Type III. The fourth set was reserved as a control, 4 drops of distilled water being added to each tube in place of the suspension of pneumococci. After incubation, inspection of the four different series showed the same result in all. As indicated in Text-fig. 1, the tubes containing the three fixed types of pneumococci showed clearing at pH 6.1 and the more alkaline end of the scale, and cloudiness in the more acid end of the scale. The control series without pneumococci showed cloudiness in the acid end of the scale due to precipitation of the bile solution at acidities greater than pH 6.1.

Microscopic examination of smears made from the cloudy tubes showed many masses of amorphous material and many pneumococci, for the most part Gram-negative, a considerable number of Gram-positive organisms, and the shadowy remains of others, while in the clear tubes only a few Gram-positive organisms were found with the shadowy remains of others.

Comparative tests with bile and standard pH solutions show that pneumococci undergo more rapid dissolution in bile. The dissolution in bile may be complete within an hour and only partial in standard solutions within this interval. Dissolution of pneumococci occurs




TEXT-FIG. 1. Dissolution of pneumococci in bile at varying hydrogen ion concentrations. Test-tubes containing 4 drops of bile at varying hydrogen ion concentrations plus 4 drops of suspension of living pneumococcus. Type I, solid line; Type II, dotted line; Type III, broken line. The observations were made after 24 hours in the incubator.

TABLE I.

*Method of Preparing Bile at Varying Hydrogen Ion Concentrations.*

| Tube No. | Bile. | Amount of HCl (half concentrated). | pH | pH after sterilization, filtration, and resterilization. |
|---|---|---|---|---|
| | | *gtt.* | | |
| 1 | Sterile bile. | 0 | 7.8 | 7.65 |
| 2 | 25 cc. of A.* | 3 | | 6.9 |
| 3 | 25 " " " | 6 | 7.3 | 6.5 |
| 4 | 25 " " " | 7 | | 6.1 |
| 5 | 25 " " B. | 6 | 5.85 | 5.5 |
| 6 | 25 " " " | 10 | 5.35 | 5.25 |
| 7 | C | 0 | 4.9 | 4.45 |

* A, fresh bile at pH 7.8 after sterilization; B, bile at pH 6.85, obtained by adding 20 drops of concentrated HCl to 100 cc. of A; C, bile at pH 4.9, obtained by adding 80 drops of concentrated HCl to 100 cc. of A.

most rapidly in the more alkaline end of the bile scale and with the lapse of time is progressive toward the more acid end, becoming complete within 24 hours at a pH of about 6.0 to 7.8 inclusive. Dissolution in standard solutions, however, takes place most quickly at a pH

of about 5.0 to 7.0 and then progresses toward the more alkaline end of the scale. As in standard solutions, dissolution in bile does not take place at the most acid end of the scale.

Dissolution of pneumococci takes place in human as well as beef bile and at the same range of pH. Pneumococci allowed to grow and die out in glucose bouillon, washed, and suspended in bile at varying pH show some but less dissolution than when living organisms are used in the experiment. Bile solubility of dead organisms takes place within the usual range of pH but more slowly than when living organisms are used.

## DISCUSSION.

The solubility of the pneumococcus in bile and in standard solutions may be ascribed to the liberation of an enzyme from the bacterial cell. The more rapid solubility of pneumococci in bile at a slightly alkaline reaction and in standard solution at a slightly acid reaction probably depends on a difference in the physical state of the bacterial cell under the influence of the two media. Bile itself probably has the property of killing the pneumococcus with a minimum of injury to the bacterial cell membrane and thus enables the endoenzymes to operate at their optimum reaction which Avery and Cullen[1] have shown to be between pH 7.0 and 7.8. Dissolution in standard solutions on the other hand, proceeds most rapidly at a slightly acid reaction because in this medium death of the organisms, which is dependent on the concentration of hydrogen ions, takes place at this reaction with a minimum of injury to the bacterial cell membrane. The more rapid dissolution in bile than in standard solutions is probably due to the more rapid death of the organisms in bile and hence the liberation of a larger amount of enzyme at the optimum pH in bile than in standard solution.

## CONCLUSION.

Dissolution of pneumococci takes place most rapidly in bile at a slightly alkaline reaction. This is probably due to death of the organisms and activation of the endocellular enzyme at its optimum hydrogen ion concentration.

[1] Avery, O. T., and Cullen, G. E., J. Exp. Med., 1920, xxxii, 547, 571, 583.

# SURFACE TENSION OF SERUM.

## II. Action of Time on the Surface Tension of Serum Solutions.

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Plates 58 to 61.

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### I.

### INTRODUCTION.

It has been shown in a previous paper[1] that the surface tension of serum decreased rapidly, as soon as the serum was exposed to the air. This phenomenon is due to the adsorption, in the surface layer, of substances which either travel slowly in the liquid, or are formed in the surface layer, or else to modifications in the arrangements of the group molecules in the surface layer, and possibly to a combination of these causes. In order to study these substances and to obtain an idea of the order of magnitude of their power of lowering the surface tension of water in function of time, it was necessary to measure the surface tension of the same samples of solutions of serum at different intervals, ranging from 2 minutes to 24 hours.

### II.

### EXPERIMENTAL.

*1. Decrease of Surface Tension of Serum Solutions in Function of Time.*—Solutions of fresh serum were made in saline solution (NaCl 0.9 per cent). The surface tension of the same layer of liquid was measured by means of du Noüy's tensiometer, according to the technique previously described.[1] In order to prevent any jarring of the samples studied, a turntable was made, supported on a ball thrust bearing, on which ten watch-glasses were placed in a circle. By

[1] du Noüy, P. L., *J. Exp. Med.*, 1922, xxxv, 575.

turning the table carefully, the watch-glasses were brought successively under the ring, and then raised smoothly by means of the screw of the instrument until the contact was established. The results obtained are shown in Table I.

Higher dilutions were prepared and tested after 1, 2, 20, and 23 hours, with the results shown in Tables II and III.

This phenomenon is illustrated by Text-fig. 1. In the charts, the value of the surface tension is taken after 2 hours arbitrarily, but the maximum drop occurs in the first 30 minutes (Text-fig. 2). The lapse of 2 hours was chosen because after that period of time, the drop is usually very slow. Text-figs. 3 and 4 show that between 1 and 2 hours the drop is small.

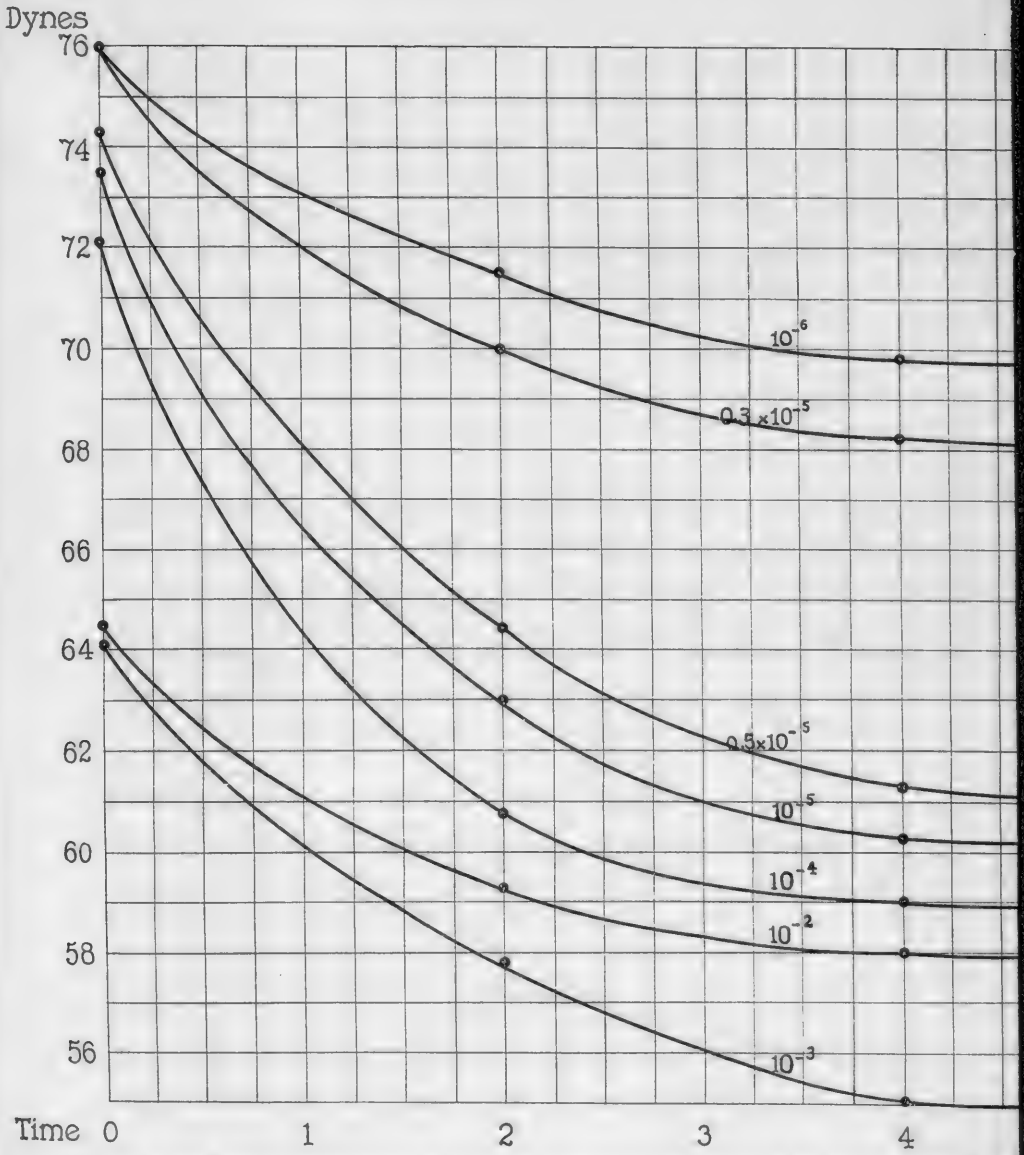
The drop in surface tension may be expressed in two different ways according to whether one chooses to take into account either the initial value of the surface tension as measured after a few seconds, or else to assume that the drop is so rapid at the beginning that a difference of 1 second or less is sufficient to affect the initial reading. In the latter case, the initial value may be taken as equal to 76 dynes, roughly, and the differences expressed thus:

$$-d\gamma = 76.0 - \gamma$$

The initial values and the values after 2 hours are shown in Text-figs. 3 to 9. The drop, $-d\gamma = \gamma_o - \gamma$, is expressed in Text-figs. 10 to 15, and the drop, $-d\gamma = 76.0 - \gamma$, in Text-fig. 16.

*2. Comparison with the Behavior of Some Organic Salts.*—It may be of interest to compare the action of serum with that of substances having a strong effect on the surface tension of water, such as sodium oleate, sodium glycocholate, and saponin, at similar dilutions in the same saline solution (Table IV). The phenomenon is illustrated in Text-figs. 17 to 19. Comparison with Text-figs. 3 to 9 shows the likeness to the behavior of serum, although the initial value is much higher in the latter case. This fact is probably due to substances which have some kind of buffing action, and oppose the lowering of surface tension of serum. The substances are particularly active when a foreign body, such as oleate or glycocholate, is added to the serum. This phenomenon will be studied in another paper.[2]

[2] du Noüy, P. L., *J. Exp. Med.*, 1922, xxxv (in press).




TEXT-FIG. 1. Values of the surface tension of serum solutions in function of the time. Serum 118 (

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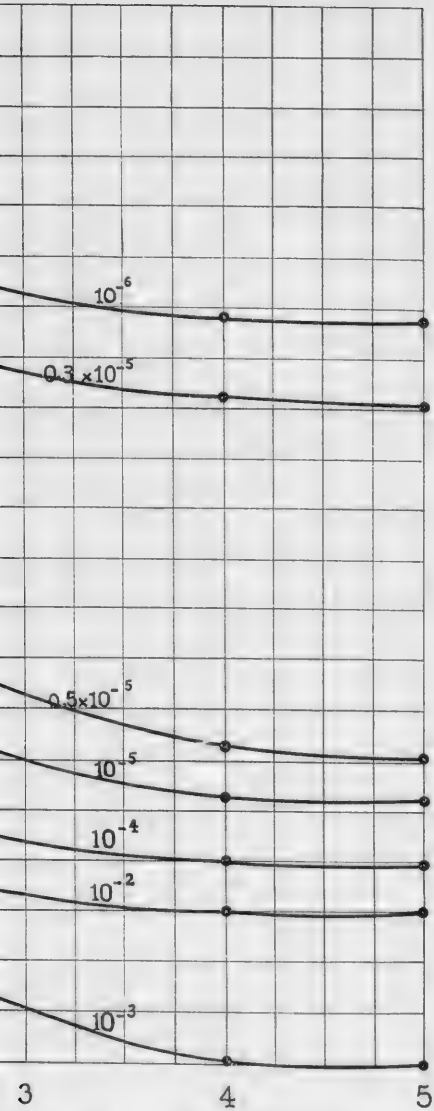
surface tension of serum solutions




in function of the time. Serum 118 (rabbit).

TABLE I.

*Decrease of Surface Tension of Serum Solutions at Different Concentrations in Function of Time.*

*Fresh Rabbit Serum, No. 114 (Text-Fig. 2).*
*Temperature 22° C.*

| Time..... | 0 | 2 min. | 6 min. | 10 min. | 30 min. | 2 hrs. | Drop in 2 hrs. |
|---|---|---|---|---|---|---|---|
| Pure. | 65.5* | 64.5 | 64.0 | 64.0 | | 63.0 | 2.5 |
| Dilution. | | | | | | | |
| 1:100 | 69.0 | 67.0 | | | 64.0 | 59.3 | 9.7 |
| 1:1,000 | 67.0 | 65.0 | | | 62.5 | 53.0 | 14.0 |
| 1:5,000 | 71.6 | 69.5 | | | 58.0 | 52.6 | 19.0 |
| 1:10,000 | 72.0 | 68.1 | 64.6 | 63.0 | 60.0 | 56.0 | 16.0 |
| 1:20,000 | 75.5 | 73.0 | | | 67.5 | 61.0 | 14.5 |
| 1:30,000 | 75.0 | 74.0 | | | 67.0 | 63.0 | 12.0 |
| 1:40,000 | 72.2 | 70.0 | | | 67.2 | 62.0 | 10.0 |
| 1:50,000 | 75.8 | 75.8 | | | 70.0 | 65.6 | 10.2 |
| 1:100,000 | 68.0 | 68.0 | | | 62.0 | 56.9 | 11.1 |
| 1:200,000 | 73.5 | 73.0 | | | 69.0 | 62.0 | 10.5 |

* In all the tables the determinations are given in dynes.

TABLE II.

*Decrease of Surface Tension of Serum Solutions at Different Concentrations in Function of Time.*

*Fresh Rabbit Serum, No. 114.*
*Temperature 22° C.*

| Time..... | 0 | 1 hr. | 2 hrs. | 20 hrs. | 23 hrs. | Drop in 23 hrs. |
|---|---|---|---|---|---|---|
| Dilution. | | | | | | |
| 1:400,000 | 67.0 | 76.2 | 67.0 | | 64.0 | 12.0 |
| 1:800,000 | 76.2 | 73.2 | 70.7 | | 67.0 | 9.2 |
| 1:1,000,000 | 76.0 | | 69.5 | 70.1 | | 6.0 |
| 1:3,000,000 | 73.5 | 71.5 | | 69.0 | | 4.5 |
| 1:5,000,000 | 72.1 | | | 70.1 | | 2.1 |
| Control. | | | | | | |
| Pure saline solution, 0.9 per cent..... | 75.3 | | | | 73.5 | 1.8 |
| 2nd sample..... | 76.5 | | | | 75.6 | 1.5 |
| 3rd " covered... | 76.2 | | | | 76.2 | 0 |

TABLE III.

*Decrease of Surface Tension of Serum Solutions at Different Concentrations in Function of Time.*

*Fresh Rabbit Serum, No. 116 (Text-Fig. 3).*

*Temperature 20° C.*

| Dilution | 1:100 | 1:1,000 | 1:10,000 | 1:100,000 | 1:1,000,000 |
|---|---|---|---|---|---|
| Initial surface tension | 68.3 | 71.0 | 73.5 | 76.0 | 76.5 |
| After 2 hrs. | 62.0 | 61.8 | 62.0 | 65.9 | 72.0 |
| " 5 " | 58.9 | 57.0 | 57.5 | 64.0 | 71.5 |

*Fresh Rabbit Serum, No. 117 (Text-Fig. 5).*

*Temperature 20° C.*

| Dilution | 1:100 | 1:1,000 | 1:10,000 | 1:100,000 | 1:1,000,000 |
|---|---|---|---|---|---|
| Initial surface tension | 66.1 | 66.5 | 72.9 | 76.0 | 76.3 |
| After 2 hrs. | 63.0 | 63.0 | 64.5 | 68.2 | 73.2 |
| " 5 " | 60.0 | 59.2 | 59.0 | 63.0 | 71.4 |

*Fresh Dog Serum, No. 1 (Text-Fig. 6).*

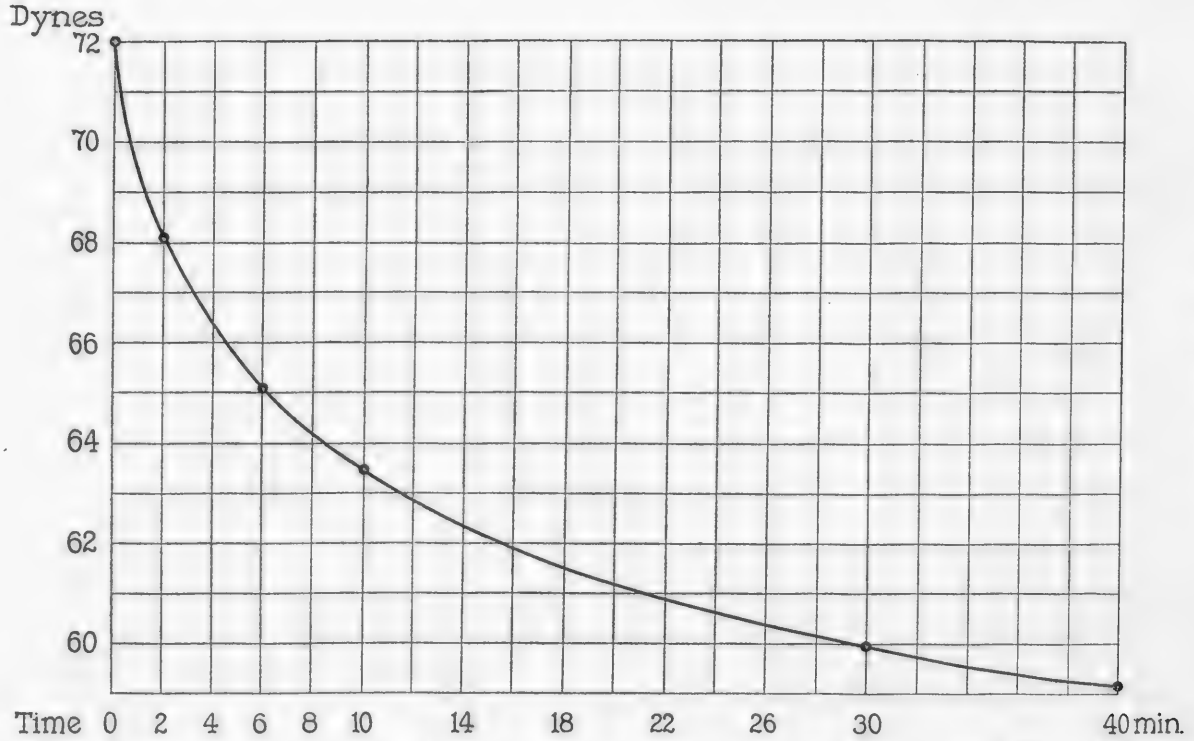
*Temperature 20° C.*

| Dilution | 1:100 | 1:1,000 | 1:10,000 | 1:100,000 | 1:1,000,000 |
|---|---|---|---|---|---|
| Initial surface tension | 63.5 | 66.0 | 73.0 | 75.0 | 76.0 |
| After 3 hrs. | 58.0 | 57.3 | 59.0 | 62.5 | 70.0 |

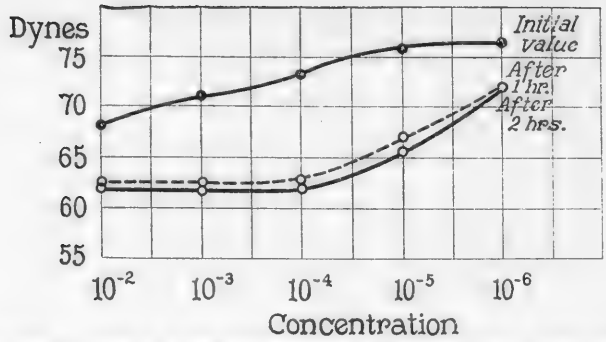
*Fresh Human Serum (Text-Fig. 8).*

*Temperature 22° C.*

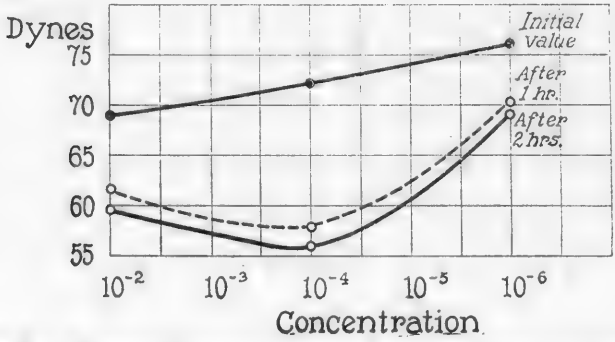
| Dilution | 1:100 | 1:1,000 | 1:10,000 | 1:100,000 | 1:1,000,000 |
|---|---|---|---|---|---|
| Initial surface tension | 61.1 | 65.0 | 72.0 | 76.0 | 76.0 |
| After 2 hrs. | 55.5 | 57.0 | 58.0 | 61.9 | 73.0 |



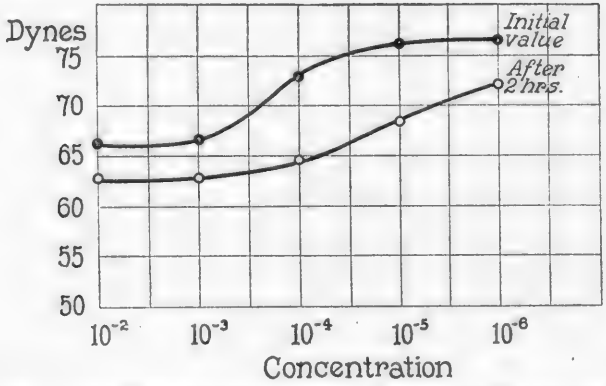

TEXT-FIG. 2. Values of the surface tension of a serum solution in the first 40 minutes. Dilution 1:10,000. Serum 114 (rabbit).




TEXT-FIG. 3. Values of the surface tension of serum solutions in function of the time. Serum 116 (rabbit).

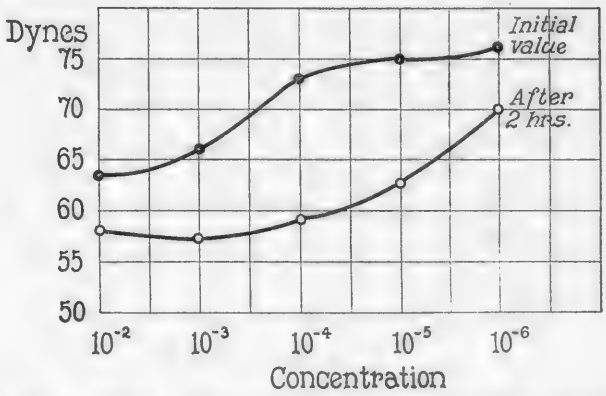



TEXT-FIG. 4. Values of the surface tension of serum solutions in function of the time. Serum 114 (rabbit).

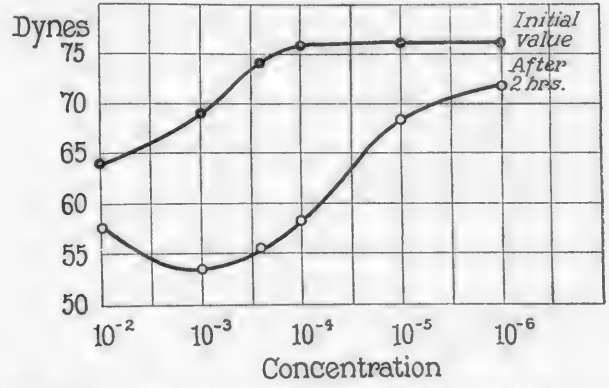



TEXT-FIG. 5. Values of the surface tension of serum solutions in function of the time. Serum 117 (rabbit).

The decrease of surface tension of solutions of organic compounds, such as glycocholate for example, follows the same law as that of serum



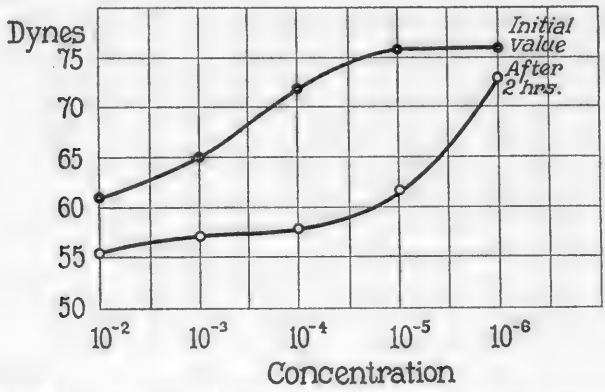

TEXT-FIG. 6. Values of the surface tension of serum solutions in function of the time. Serum 1 (dog).



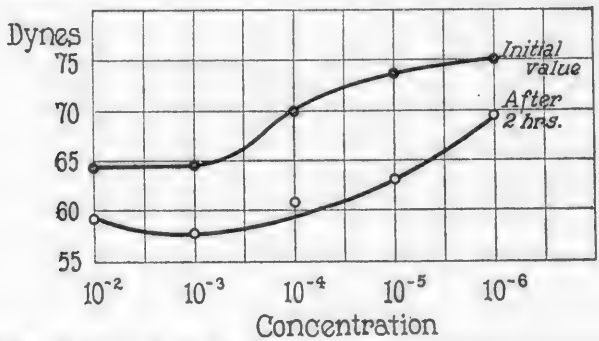

TEXT-FIG. 7. Values of the surface tension of serum solutions in function of the time. Young dog serum.

and is also very rapid at the beginning (Text-fig. 20). In this case the glycocholate was dissolved in distilled water (concentration $10^{-4}$). When serum is diluted in distilled water, the same phenomenon occurs

(Text-figs. 21 and 22). The maxima and the comparison with serum are shown in Text-figs. 23 and 24.




TEXT-FIG. 8. Values of the surface tension of serum solutions in function of the time. Human serum.

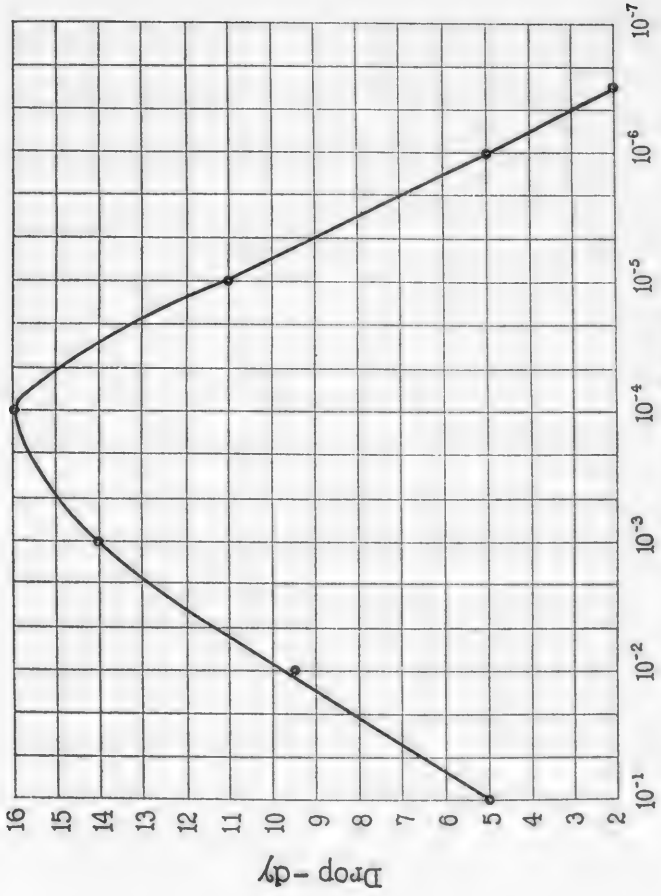



TEXT-FIG. 9. Values of the surface tension of serum solutions in function of the time. Serum 118 (rabbit).

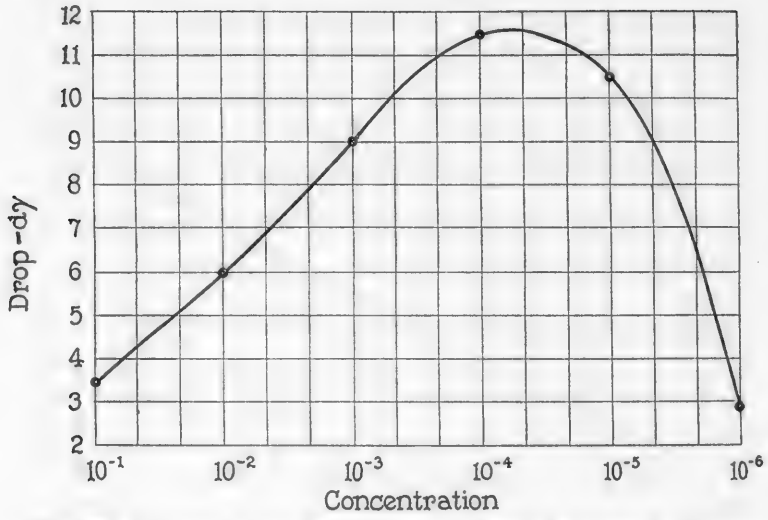
It may be of interest to refer the reader to the work of Berczeller[3] on the surface tension of colloidal solutions and, in general, to the numerous papers published by Traube on the same subject. The book by Freundlich,[4] a great number of papers

[3] Berczeller, L., *Biochem. Z.*, 1913, liii, 215; 1914, lxvi, 173; *Internat. Z. physik.-chem. Biol.*, 1914, i, 124.

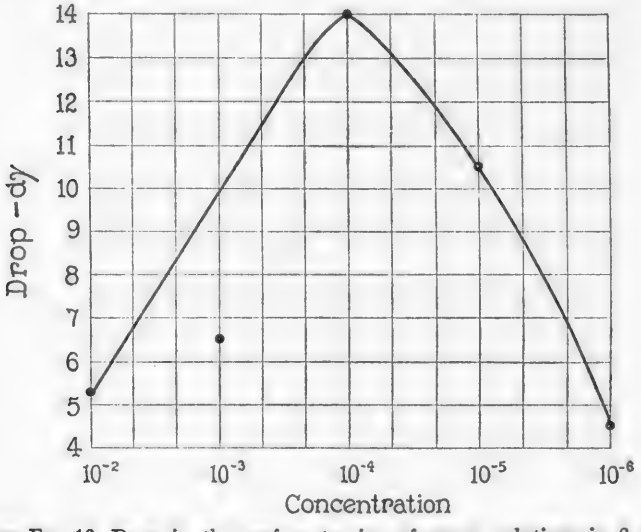
[4] Freundlich, H., Kapillarchemie: Eine Darstellung der Chemie der Kolloide und verwandter Gebiete, Leipsic, 1909.



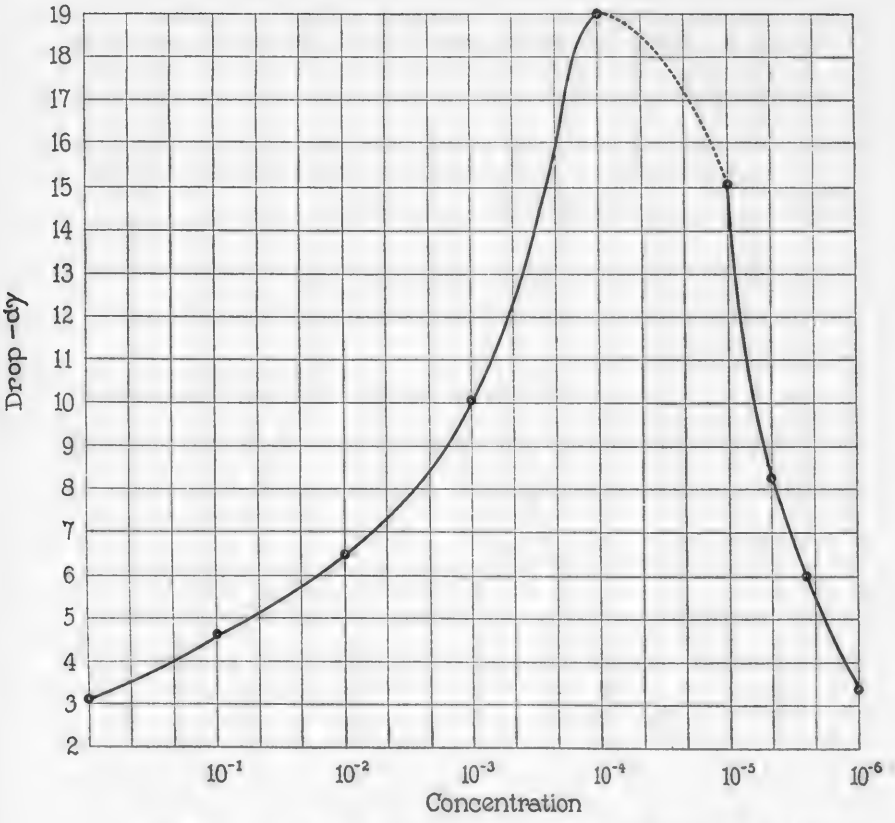

TEXT-FIG. 10. Drop in the surface tension of serum solutions in 2 hours. Serum 114 (rabbit).




TEXT-FIG. 11. Drop in the surface tension of serum solutions in 2 hours. Serum 116 (rabbit).

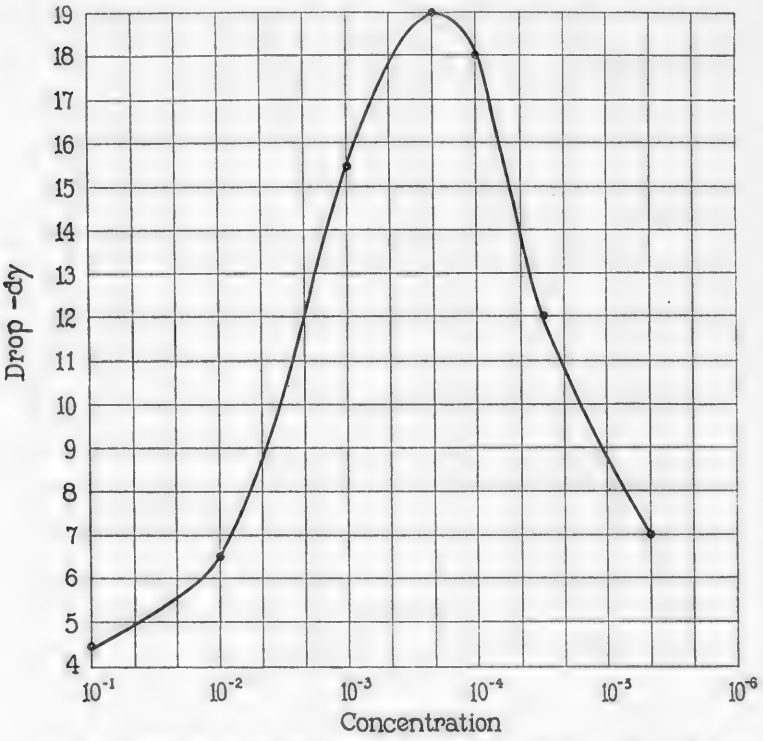



TEXT-FIG. 12. Drop in the surface tension of serum solutions in 2 hours. Serum 118 (rabbit).




TEXT-FIG. 13. Drop in the surface tension of serum solutions in 2 hours. Old dog serum. As no measurements were made at concentrations between $10^{-4}$ and $10^{-5}$ a dotted line is used to connect these two points, since it is probable that the maximum would have been somewhere between these two concentrations. Therefore it is likely that the dotted line does not represent the exact curve.

in the *Internationale Zeitschrift für physikalisch-chemische Biologie*, and the papers by Posternak[5] are also worth perusal. It seems that the solution of the problem has been postponed mainly by the confusion of the action of time with that of heat and of chemical reactions. Therefore, it is important to determine, in all studies on colloidal solutions, the part due to their colloidal state and that which may be due to really specific actions (for instance the inactivation of serum by heating at 56° C.).

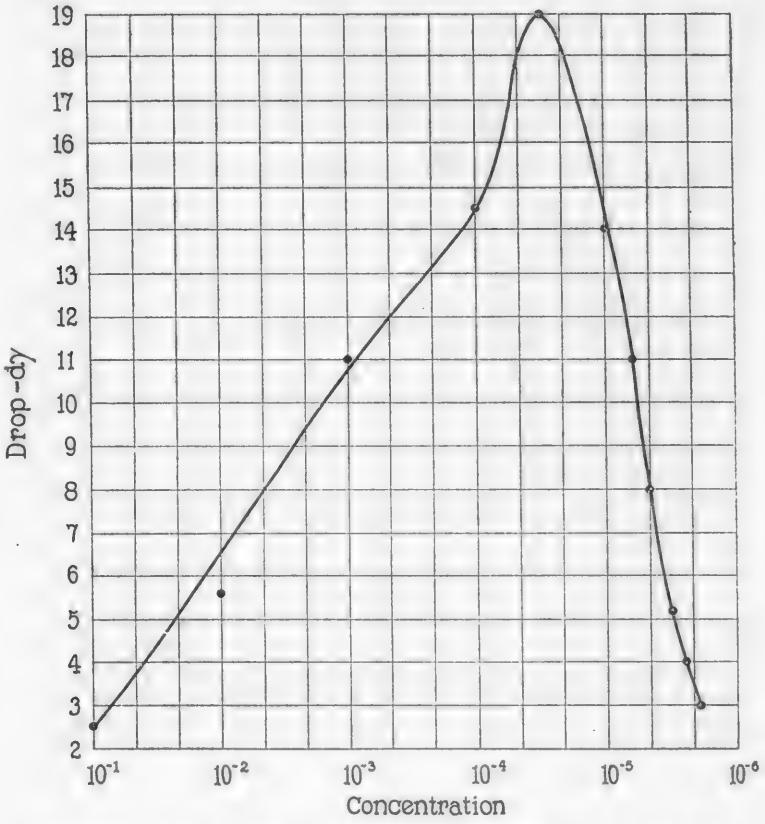



TEXT-FIG. 14. Drop in the surface tension of serum solutions in 2 hours. Young dog serum.

Thus, by merely introducing the factor time, are explained some of the discrepancies hitherto unaccounted for between the values of surface tension of solutions of organic compounds, as given by static

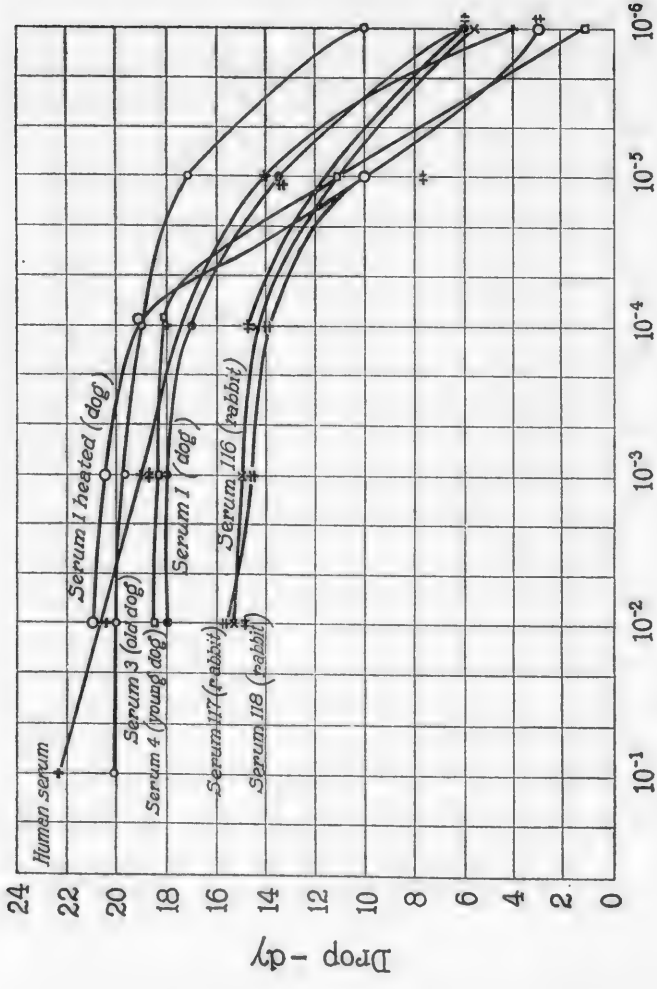
[5] Posternak, S., *Ann. Inst. Pasteur*, 1901, xv, 85, 169, 451, 570.

and dynamic methods. Indeed, certain methods, such as the capillary ascension for instance, measure the surface tension of the same layer of liquid in which adsorption in function of the time takes place, while




TEXT-FIG. 15. Drop in the surface tension of serum solutions in 2 hours. Human serum.

most dynamic methods, such as the undulatory jet methods, deal with a continuously renewed surface. The drop weight method is intermediate, the drops requiring a longer time to fall. It is quite obvious that none of these last methods could show the drop in




TEXT-FIG. 16. Drop in the surface tension of solutions of different sera in 2 hours. Value after 2 hours subtracted from 76.0 dynes.

TABLE IV.

*Decrease of Surface Tension of Solutions of Sodium Oleate, Sodium Glycocholate, and Saponin in a 0.9 Per Cent Saline Solution in Function of Time.*

*Sodium Oleate (Text -Fig. 17).*

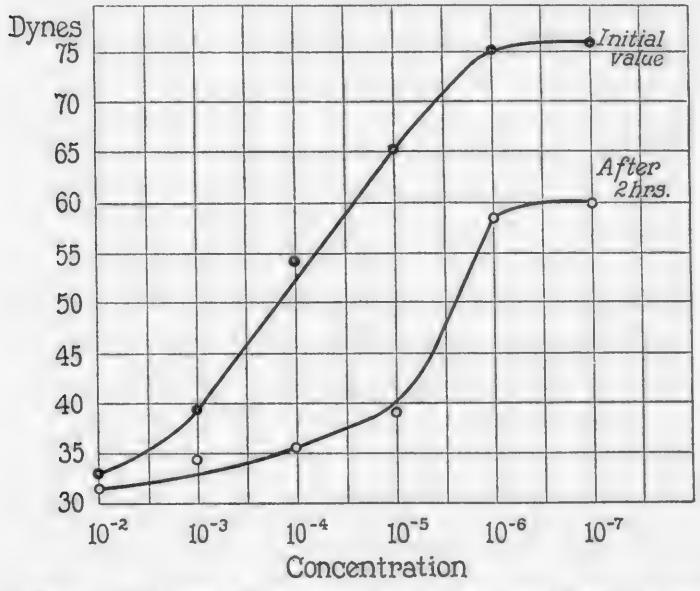
| Dilution | 1:100 | 1:1,000 | 1:10,000 | 1:100,000 | 1:1,000,000 |
|---|---|---|---|---|---|
| Initial surface tension | 33.2 | 39.5 | 54.2 | 59.1 | 75.1 |
| After 2 hrs | 31.5 | 34.5 | 35.5 | 39.0 | 58.6 |

*Sodium Glycocholate (Text-Fig. 18).*

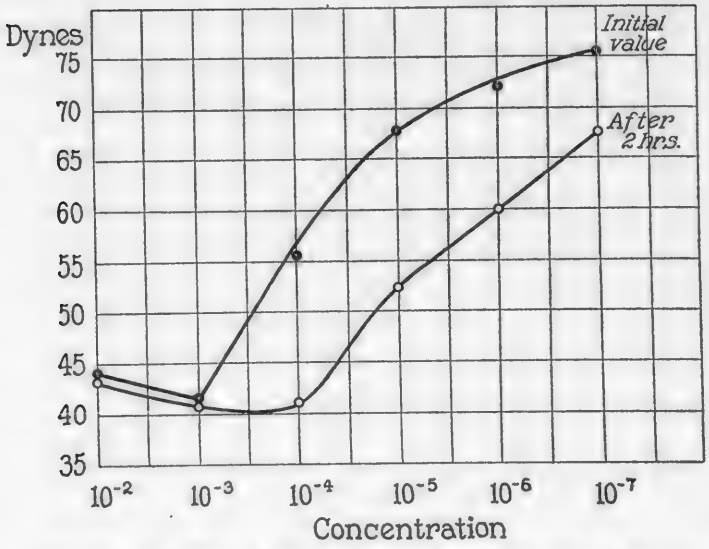
| Dilution | 1:100 | 1:1,000 | 1:10,000 | 1:100,000 | 1:1,000,000 |
|---|---|---|---|---|---|
| Initial surface tension | 44.0 | 41.5 | 55.6 | 68.0 | 72.0 |
| After 2 hrs | 43.4 | 41.0 | 41.5 | 60.6 | 60.0 |

*Saponin (Text-Fig. 19).*

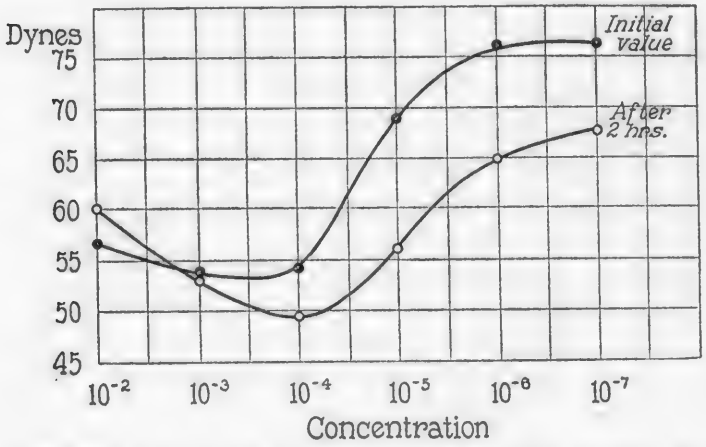
| Dilution | 1:100 | 1:1,000 | 1:10,000 | 1:100,000 | 1:1,000,000 |
|---|---|---|---|---|---|
| Initial surface tension | 56.8 | 54.0 | 54.2 | 69.0 | 76.0 |
| After 2 hrs | 60.5 | 53.5 | 49.5 | 56.0 | 65.0 |



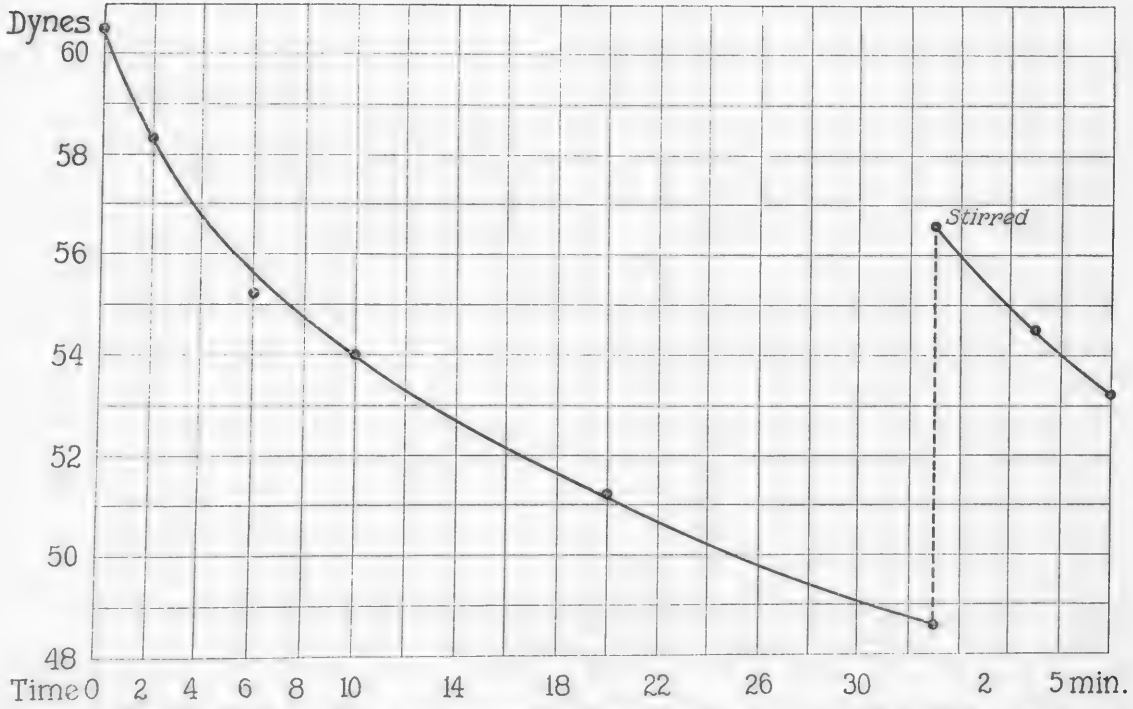

TEXT-FIG. 17. Values of the surface tension of solutions of sodium oleate in saline solution.



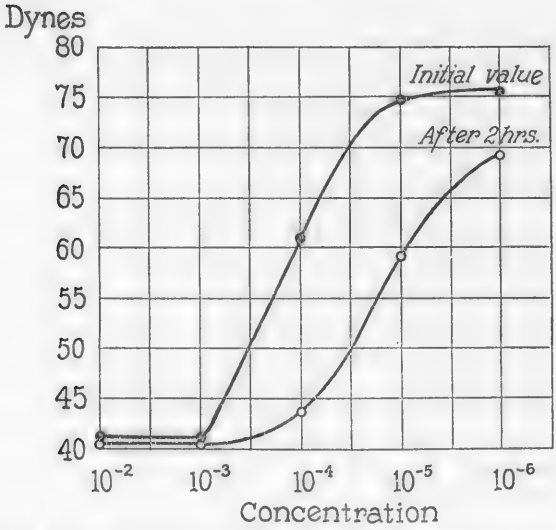

TEXT-FIG. 18. Values of the surface tension of solutions of sodium glycocholate in saline solution.



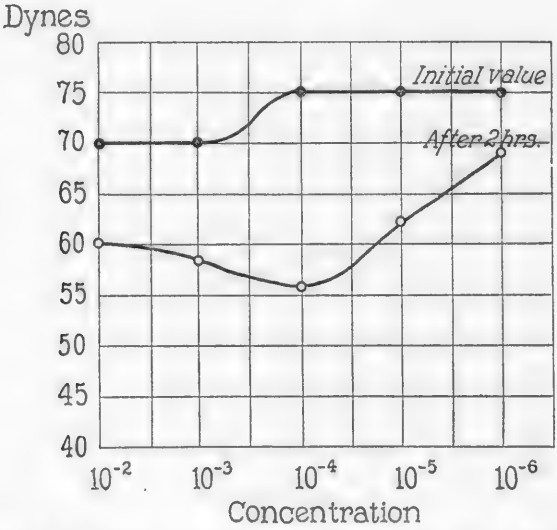

TEXT-FIG. 19. Values of the surface tension of solutions of saponin in saline solution.




TEXT-FIG. 20. Values of the surface tension of solutions of sodium glycocholate in distilled water. Drop in the first 30 minutes; effect of stirring. Concentration 1:10,000.

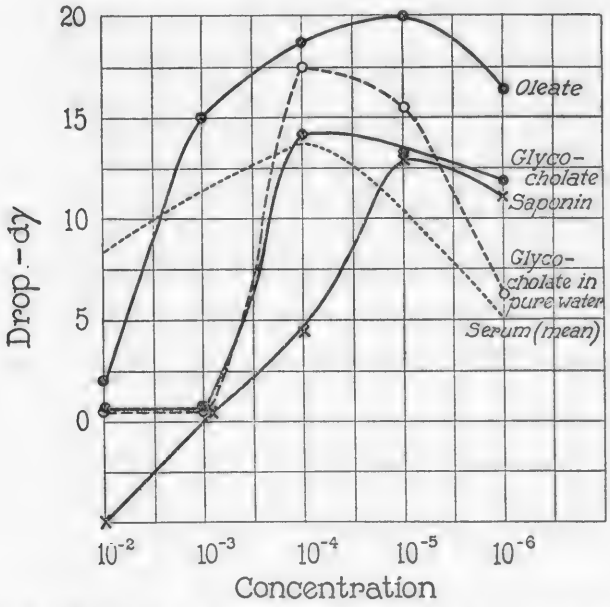



TEXT-FIG. 21. Values of the surface tension of solutions of sodium glycocholate in distilled water.




TEXT-FIG. 22. Values of the surface tension of solutions of serum in distilled water. Serum 118 (rabbit).

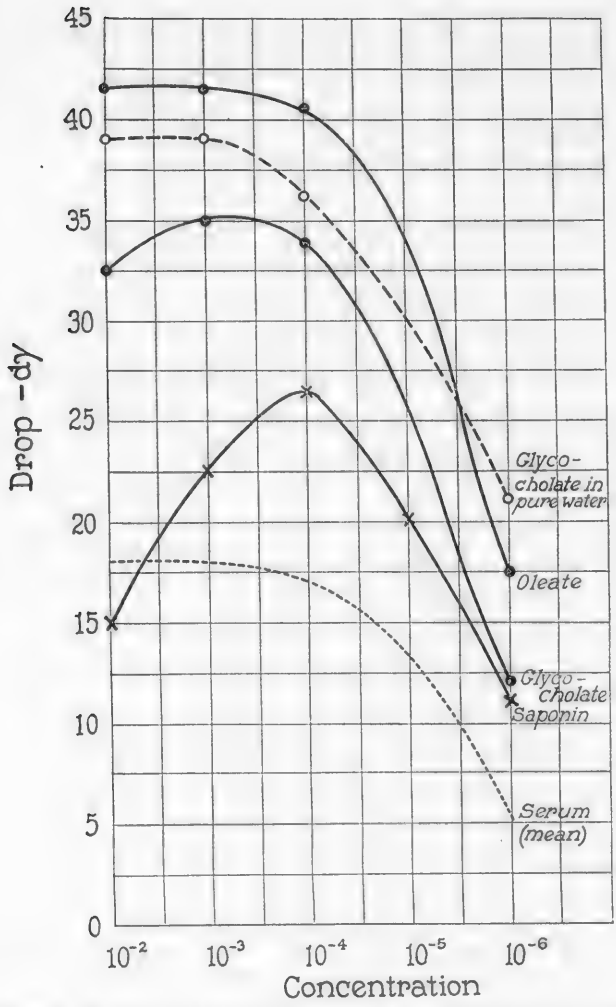
surface tension which occurs after a few minutes. With a solution of 1:100,000 the number of drops was the same as for pure saline solution: 17 drops whenever the measurement was made with a Traube stalagmometer (after a few seconds, 10 minutes, 2 hours, or 24 hours). The tensiometer measured for the same intervals: 75.5, 66.0, 61.0, 55.5 dynes; that is, a drop of 20 dynes in 24 hours.




TEXT-FIG. 23. Drop in the surface tension of different solutions as expressed by the formula

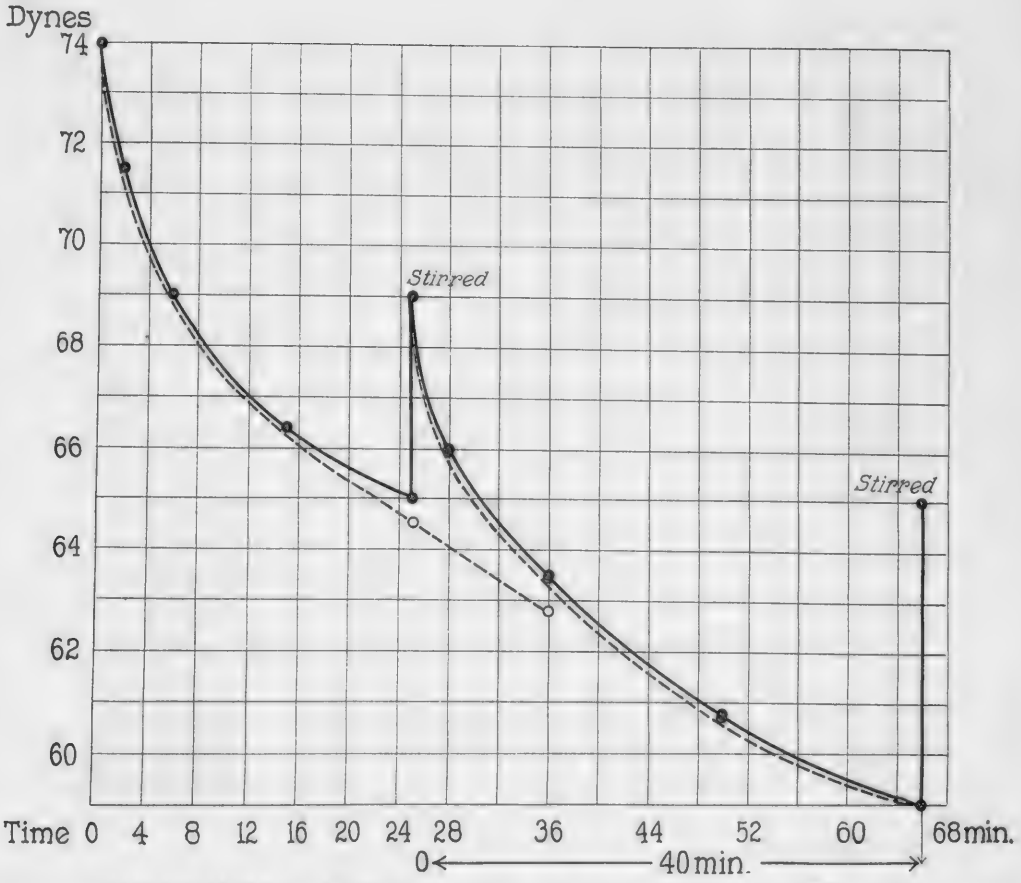
$$-d\gamma = \gamma_o - \gamma$$

*3. Effect of Stirring.*—When stirred, these solutions present the same phenomenon as the pure serum; the surface tension rises and sometimes reaches the same value as at the beginning of the experiment (Table V and Text-figs. 25 and 26). When the solutions are kept in test-tubes, that is with a relatively small free surface, vigorous stirring after 24 hours brings the surface tension to the same value (Table VI). This seems to indicate that the damping effect,



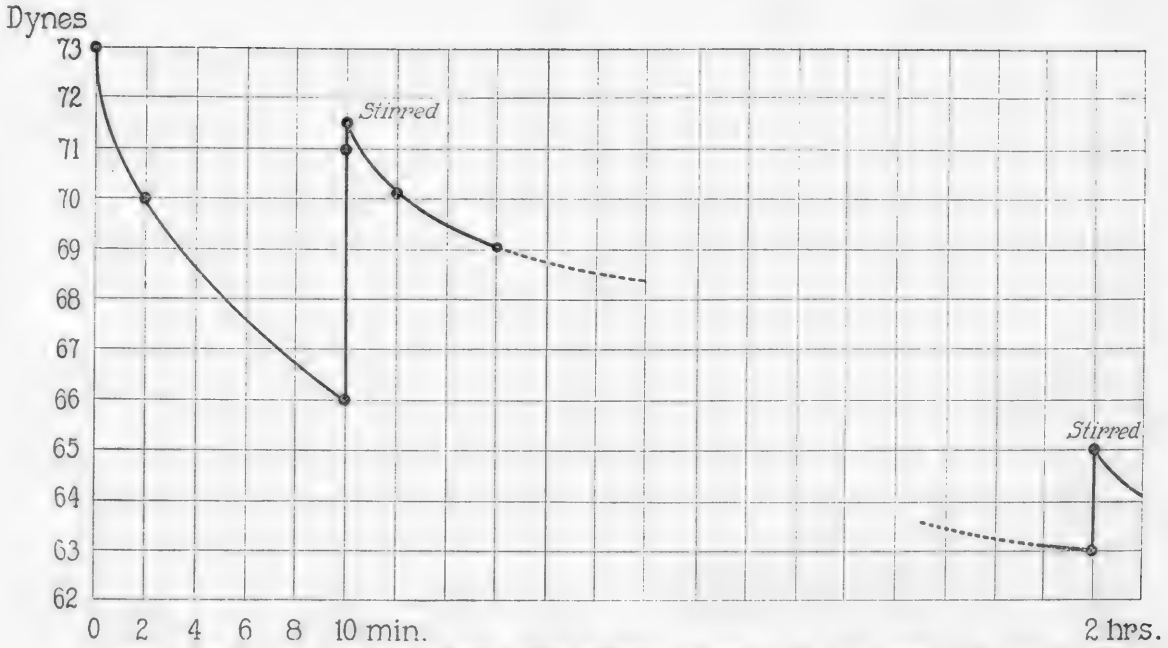

TEXT-FIG. 24. Drop in the surface tension of different solutions as expressed by the formula

$$-d\gamma = 76-\gamma$$




TEXT-FIG. 25. Effect of stirring on solutions of serum. Dilution 1:10,000. Serum 2 (dog). The dotted line is calculated according to the formula

$$\gamma = \gamma_0 e^{-Kt^{\frac{1}{3}}}$$




TEXT-FIG. 26. Effect of stirring on solutions of serum. Dilution 1:10,000. Serum 112 (rabbit). The interruption of the curve after the 22nd minute was necessary to bring the end of the curve after 2 hours within the same chart, without making it too long.

TABLE V.

*Effect of Stirring on the Surface Tension of Serum Solutions.*

*Dog Serum, No. 2 (Text-Fig. 25).*

*Temperature 22° C.*

| Time | 0 | 25 min. | Stirred. | 40 min. | Stirred. |
|---|---|---|---|---|---|
| Dilution. | | | | | |
| 1:10,000 | 74.0 | 64.9 | 69.0 | 59.0 | 65.0 |

*Rabbit Serum, No. 112 (Text-Fig. 26).*

*Temperature 22° C.*

| Time | 0 | 10 min. | Stirred. | 2 hrs. | Stirred. |
|---|---|---|---|---|---|
| Dilution. | | | | | |
| 1:10,000 | 73.0 | 66.0 | 71.5 | 63.0 | 65.0 |

*Rabbit Serum, No. 118.*

*Temperature 23° C.*

| Time | 0 | 24 hrs. | Stirred vigorously. |
|---|---|---|---|
| Dilution. | | | |
| 1:10,000 | 76.0 | 68.2 | 75.1 |

TABLE VI.

*Effect of Stirring on the Surface Tension of Serum Solutions.*

*Dog Serum, No. 3, Kept in Test-Tubes.*

*Temperature 23° C.*

| Time | 0 | 24 hrs., stirred vigorously. |
|---|---|---|
| Dilution. | | |
| 1:10 | 61.9 | 62.0 |
| 1:100 | 62.5 | 60.0 |
| 1:1,000 | 66.2 | 66.0 |
| 1:10,000 | 76.0 | 76.0 |
| 1:100,000 | 74.0 | 74.0 |

described in our previous paper,[6] is due to chemical changes undergone in the surface layer. When the surface layer is small with respect to the bulk, the changes are undetectable.

## III.

### DISCUSSION.

*1. Interpretation of the Maxima.*—From Table I, as well as from Text-figs. 10 to 15, it is clear that the drop in the value of the surface tension is variable and reaches a maximum at a dilution of about 1:10,000. In general, this occurs only when the measured initial value of the tension of the serum is taken into consideration. In other words, if we assume the initial value of the tension to be around 76 dynes, as in the higher dilutions, there would not always be a maximum. Therefore, we assume provisionally that the actually initial surface tension of such dilutions as 1:10, 1:100, 1:1,000, 1:10,000, is that which can be measured within 5 or 10 seconds after the liquid is poured into the watch-glass. It is very likely that there are at least two groups of substances which act powerfully upon the surface tension of the serum, and in different ways. The first group, in conformity with the substances already known, is adsorbed almost instantaneously in the surface layer, and determines the value of the surface tension of fresh serum and of serum solutions up to about 1:50,000, as given by the drop weight methods (with continuously renewed free surfaces); the other group which, on account of the techniques in use up to the present time, could not be detected is adsorbed in function of time, provided the free surface is left unstirred. This second substance, or group of substances, acts more powerfully than the other, and at extremely low concentrations. Indeed, we have seen that one-millionth part of serum is still active. Since serum does not usually contain as much as 10 per cent of solid substances in solution altogether, we may assume, and this is manifestly exaggerated, that the serum contains 5 per cent of these substances. Hence, this would mean that they are still active at such a low concentration as 1:20,000,000 (1 gm. in 20,000 liters, or over 5,200 gallons of water). The hypothesis of the two groups of sub-

[6] du Noüy,[1] p. 579.

stances is expressed by Text-figs. 10 to 15, in which the initial value of the surface tension has been taken into consideration for the evaluation of the drop. By assuming that it is the same substance, or group of substances, which determines the initial value of the surface tension by very rapid adsorption, and the slow drop by slower adsorption, we obtain Text-fig. 16.

Hence, admitting the correctness of the assumption of the existence of two groups of substances, it becomes easy to account for the special grouping of the initial values of surface tension. Indeed, if we call the first group A, and the second group B, it is readily seen, by looking at the charts, that Group A, present at dilutions from 1:10 to 1:1,000 in sufficient quantities to have a marked action on the tension, is no longer effective in the case of high dilutions, from 1:100,000 up. There is a break in the initial value which is not always bridged by the 1:10,000 dilution. From 1:100,000 up, practically only Group B is active. On the other hand, we notice that it is precisely around the concentration of 1:10,000 that the drop is maximum. The reason is simple if we refer to Langmuir's ideas on the structure of the surface layer of solutions, when the solute is adsorbed in the surface layer. Langmuir[7] has shown, indeed, that the free surface of such a liquid is generally composed of a monomolecular layer in which the molecules are geometrically disposed and similarly oriented with reference to a three dimensional space; in other words, that the surface of the liquid is composed of a sort of mosaic, each element of which is the same end of the same side of the molecules of the liquid. Furthermore, he states that the group molecules of organic liquids arrange themselves in such a way that their active portion is drawn inwards, leaving the least active portion of the molecule to form the surface layer. This hypothesis is supported by very striking facts, but their description would be beyond the scope of this paper.

Thus, in the case of serum solutions, the maximum drop would correspond to such a dilution as would allow the active molecules of Group B to dispose themselves with what is left of Group A in the most favorable way for the decrease of surface tension; and this is only possible at one precise concentration. At higher concentrations, the

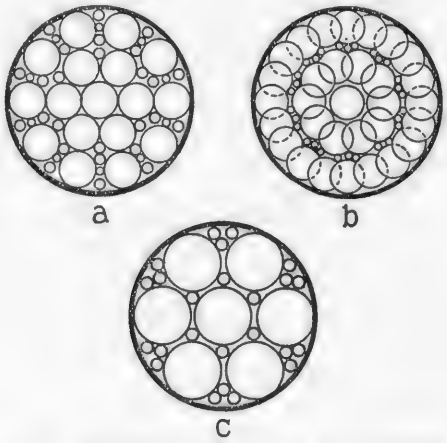
[7] Langmuir, I., *Met. Chem. Eng.*, 1916, xv, 468; *J. Am. Chem. Soc.*, 1917, xxxix, 1848.

excess of molecules of Group A interferes with the best possible geometrical arrangement of the molecules of Group B, and the forcing apart of these groups increases the surface tension. The same thing happens at higher dilutions, in which the forcing apart is due to the smaller number of molecules of Group B, which can no longer cover the liquid completely. This is, of course, a mere hypothesis which has, at least, the advantage of accounting for the facts found so far. But, whatever hypothesis is accepted, and in spite of the fact that the maximum drop is not so clear if the presence of only one group of substances acting together in the same way is assumed, there is no doubt but that an optimum concentration exists; it is easily explained, as well as the grouping of the initial values, on the basis of Langmuir's conception of the structure of liquid layers.

A glance at the charts will also show that the maximum drop does not always occur at the same concentration. Assuming the correctness of our hypothesis, this shift may be due to a change in the size of the group molecules. Indeed, the maximum drop occurs when the free surface is entirely covered with a certain lattice of molecules of Groups A and B, arranged in such a way as to produce the weakest force fields. Evidently there is an optimum concentration, as has been said before. But this optimum is function of the size of the molecules, and of the relative concentration of the two groups, A and B. This will be more clearly understood by looking at Text-fig. 27, *a* to *c*. In Text-fig. 27, *a*, the optimum is attained for a certain size of the molecules, and a certain relative number of both groups. The quantity adsorbed in the surface layer is exactly that which corresponds to the minimum surface tension. In Text-fig. 27, *b*, the size of the molecules is the same and the quantity adsorbed is greater, the concentration being higher. The arrangement of the molecule is no longer perfect, the proportion of Groups A and B is changed, and the molecules may be forced to tilt on one side, or to overlap. The result is that the surface tension is increased. In Text-fig. 27, *c*, we have another optimum arrangement, with a smaller number of larger molecules; that is, at a lower concentration. Although this illustration is very crude, it helps to visualize a plausible process through which the maximum may be shifted. As the molecules in solution are probably very long, with lateral branches, instead of

discs or spheres, as we have symbolically represented them, these figures must not be taken as an image of reality but as a mere comparison.

In the curves published, the maximum for human serum lies at 1:50,000, for the old dog serum at 1:30,000, for rabbit serum around 1:10,000, and for a very young pup at 1:7,000.




TEXT-FIG. 27, *a* to *c*. Hypothetical illustration of the possible arrangement of molecules in the surface layer of a serum solution.

IV.

*Aspect of Crystallization of Serum Solutions.*

When the solutions are allowed to evaporate, the sodium chloride crystallizes. By using watch-glasses, an interesting phenomenon is observed. When a pure sodium chloride solution crystallizes, it concentrates first, then the crystals are formed at the bottom of the glass as seen in Fig. 1. But when serum is added, very small crystals spread all over the watch-glass, and assume different aspects, according to the concentration, as shown by Figs. 2 and 3. Up to a concentration of 1:500,000 in the case of Serum 114, the effect of the lowering of the surface tension can be detected easily by the action of the substances adsorbed in the surface layer upon the molecules of sodium chloride. This phenomenon can be explained in the

following way. The large adsorbed molecules carry with them in the surface layer the Na and Cl ions. The concentration there becomes very high, and dissociation small. Sodium chloride molecules are formed and when the liquid evaporates, it progressively abandons the salt on the glass. No concentration occurs in the bulk, and no large crystals can grow at the bottom.

The phenomenon is similar when other surface-active substances are used, such as for instance saponin (Fig. 4).

This phenomenon is interesting because it shows that the presence of sodium glycocholate or taurocholate or dried serum in such a high dilution as 1:10,000,000 modifies profoundly the state of equilibrium and the distribution of phases in a liquid containing crystalloids in solution. Gibbs' statement that substances which increase the surface tension are more concentrated in the bulk is no longer true in this case. The birth of the membranes at interfaces, the first manifestation of the individuality of a cell, becomes very clear. The concentration of colloids and salts being much greater in the surface layer, precipitation will naturally occur there, and electric phenomena are forcibly different from those in the bulk. The aspect of the crystals is not the same at all dilutions, and at a concentration of 1:100, all sera studied so far have shown a very peculiar phenomenon, easily seen in the photographs. The more or less regular rings cannot be found as clearly at any other concentration. The white rings are very small sodium chloride crystals. The theoretical explanation of this phenomenon is not obvious. There is no doubt that it corresponds to different states of equilibrium of the surface layer, and possibly to different and periodical changes in its surface tension. This phenomenon is somewhat similar to that observed by Liesegang,[8] obtained by adding 1 drop of a silver nitrate solution to a solution of gelatin and potassium dichromate.

As was to be expected, sodium oleate, glycocholate, and saponin act in the same way. Further investigations are being conducted on this subject.

[8] Liesegang, R. E., *Z. Chem. u. Indust. Kolloide*, 1907–08, ii, 70.

## V.

### CONCLUSIONS.

The application of the ring method to the measurement of solutions of serum and of certain organic compounds has brought forth new facts, mainly the decrease of the surface tension of such solutions in function of time.

1. In serum diluted at such a low concentration as 1:1,000,000 in NaCl, physiological solution, the surface tension of the liquid is lowered by 3 or 4 dynes in 2 hours; at 1:100,000, by about 11 dynes (mean value) in 2 hours, and by 20 dynes in 24 hours; at 1:10,000 by about 13 to 16 dynes in 2 hours.

2. The drop in surface tension is much more rapid in the first 30 minutes and follows generally the law of adsorption in the surface layer in function of the time.

3. Stirring or shaking after the drop causes the surface tension to rise, but generally below its initial value.

4. The same phenomena occur when using sodium oleate, glycocholate, or saponin instead of serum.

5. For every serum, as well as for the substances mentioned above a maximum drop occurs in certain conditions at a given optimum concentration.

6. Not only are the substances which lower the surface tension adsorbed in the surface layer, in the case in which they are present with crystalloids, but also the crystalloids themselves, in contradiction to Gibbs' statement. This is plainly shown by the evaporation of such solutions in watch-glasses which, instead of a small group of sharp, large, well defined crystals at the bottom, leaves a white disc almost as large as the initial free surface itself, due to the liberation of the salt by the surface layer as it crawls down the concave surface of the glass.

7. In these conditions, solutions of serum are characterized by a very peculiar periodic and concentric distribution of the crystals, at a concentration of 1:100 only. The same ring-like aspect is observed with sodium oleate, glycocholate, and saponin, but not at the same concentration, as was to be expected, since serum is a solution in itself.

## EXPLANATION OF PLATES.

### PLATE 58.

FIG. 1. Crystals of serum solutions in NaCl solution at 9 per cent. Serum 114 (rabbit).

### PLATE 59.

FIG. 2. Crystals of serum solutions in NaCl solution at 9 per cent. Serum 117 (rabbit).

### PLATE 60.

FIG. 3. Crystals of serum solutions in NaCl solution at 9 per cent. Human serum.

### PLATE 61.

FIG. 4. Crystals of saponin solutions in NaCl solution at 9 per cent.

On page 735, Vol. xxxv, No. 5, Figs. 1, 2, 3, and 4, for *NaCl solution at 9 per cent* read *NaCl solution at 0.9 per cent.* On Plate 58 of the same article, at the lower left hand corner, for *NaCl 9 per cent* read *NaCl 0.9 per cent.*

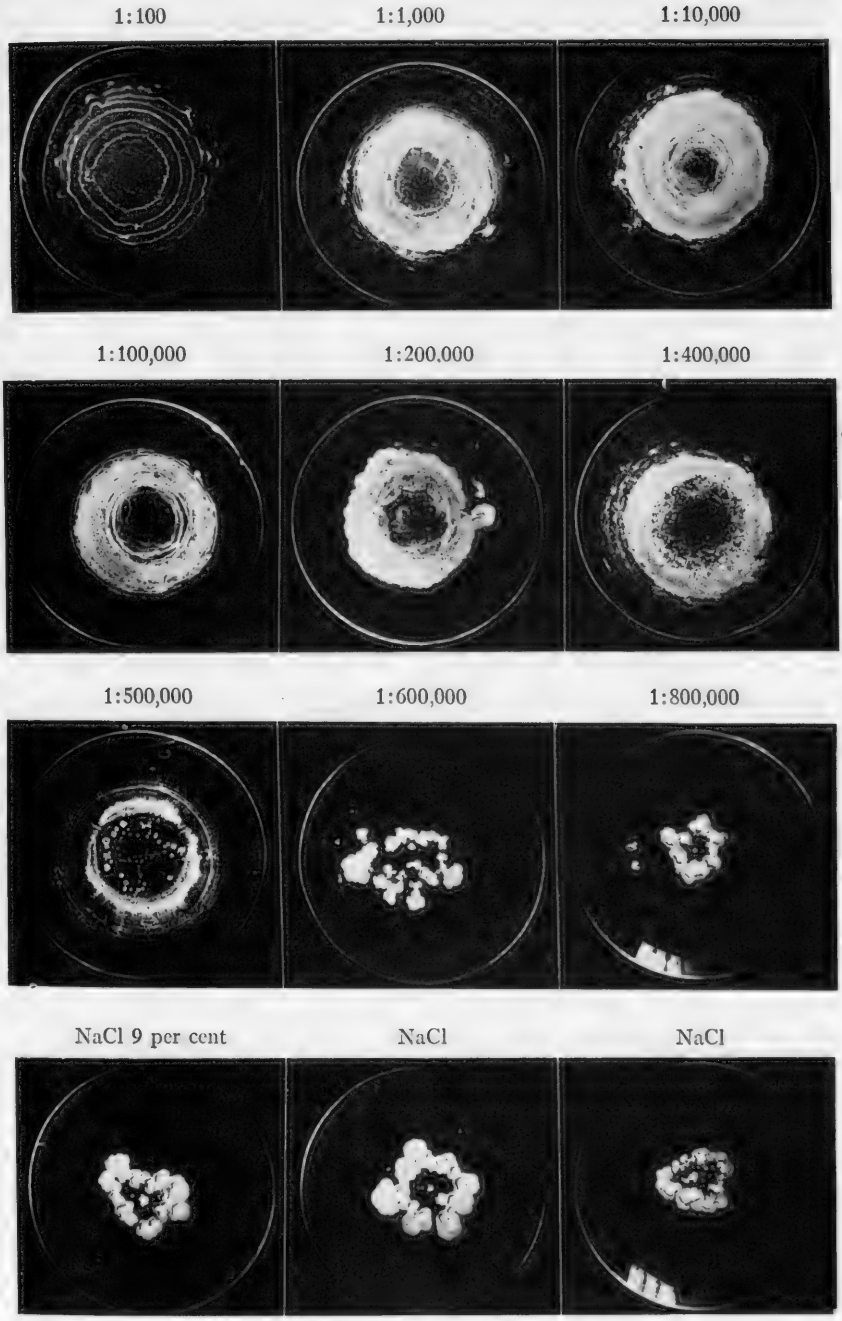



FIG. 1.

(du Noüy: Surface tension of serum. II.)

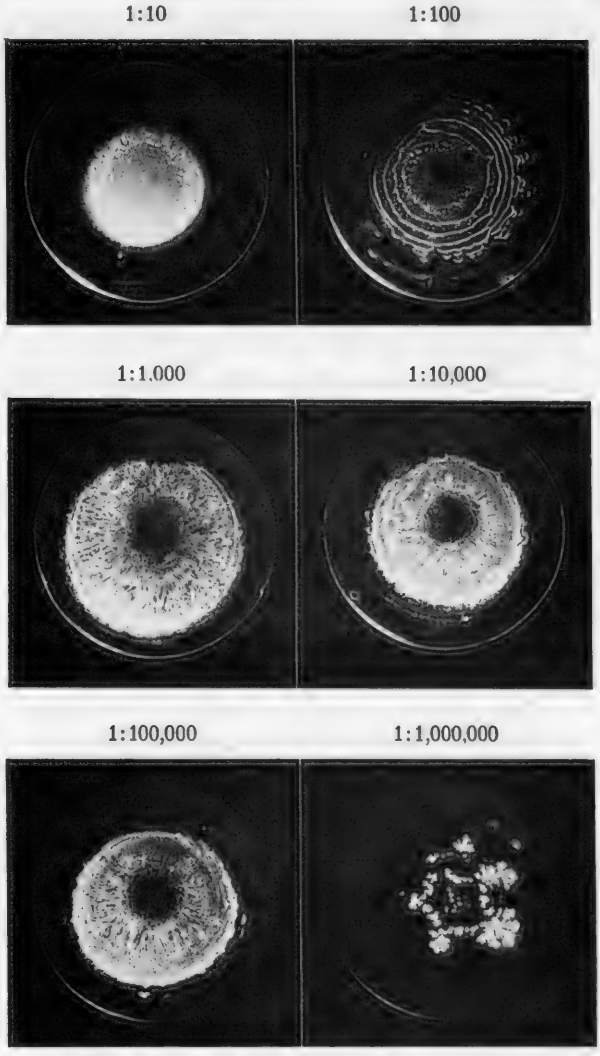



FIG. 2.

(du Noüy: Surface tension of serum. II.)

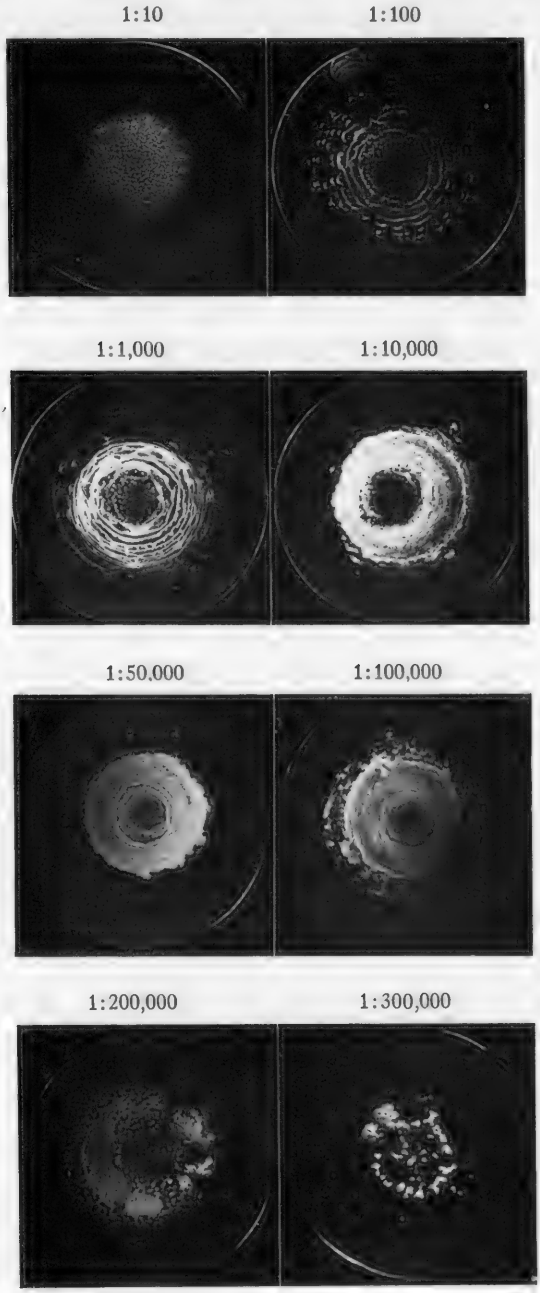



FIG. 3.

(du Noüy: Surface tension of serum. II.)

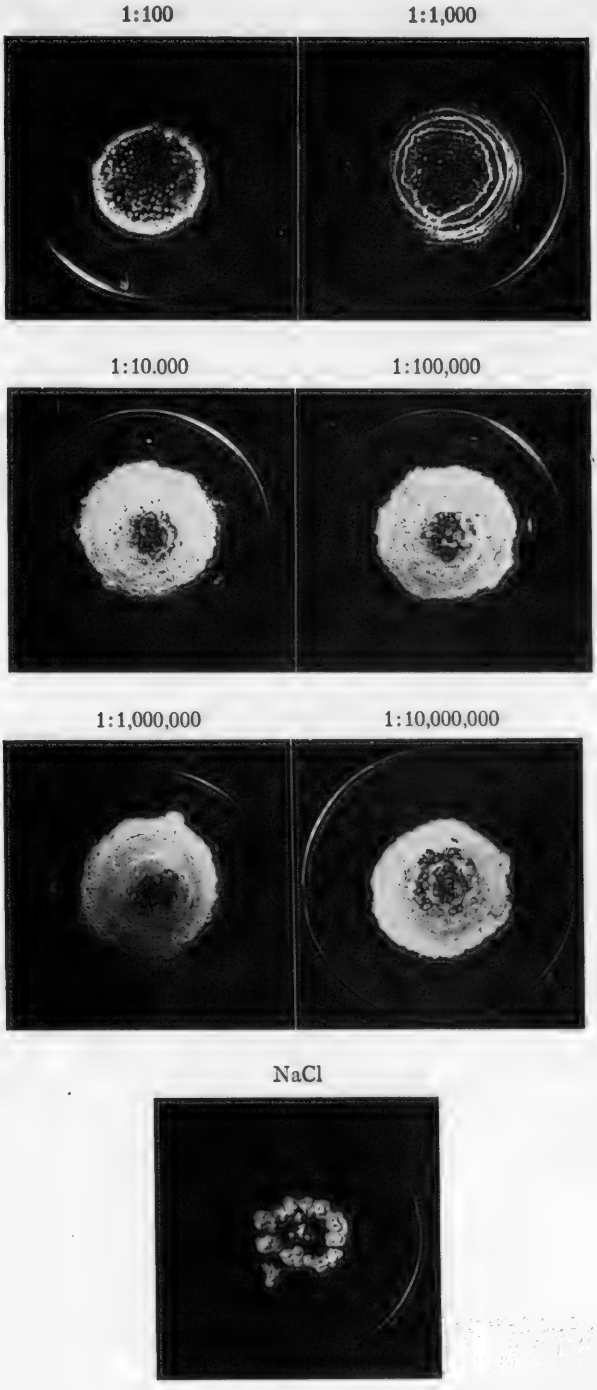



FIG. 4.

(du Noüy: Surface tension of serum. II.)

# PATHOLOGY OF THE DERMATITIS CAUSED BY MEGALOPYGE OPERCULARIS, A TEXAN CATERPILLAR.*

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PLATES 62 AND 63.

(Received for publication, December 22, 1921.)

## INTRODUCTION.

A small larval moth has been causing dermatitis in southern Texas for the past decade, particularly during the years 1913 and 1920. It is known locally as the puss-caterpillar, opossum bug, Italian asp, and, by Mexicans, *el perrito* (the puppy), and belongs to the family Megalopygidæ, of the group known as slug caterpillars. As it has not been investigated systematically from a medical standpoint up to the present time, the work reported in this paper was undertaken at the suggestion of Mr. F. C. Bishopp, of the United States Bureau of Entomology, who is stationed at Dallas, Texas, and to whom the writer is much indebted for the caterpillars studied and notes concerning their distribution and injurious effects.

Mr. Bishopp reports the following clinical symptoms from regions infested by the caterpillars. All stages of the larva are capable of inflicting stings, the intensity of the lesions being in proportion to the size of the caterpillar. There is a marked variability in the susceptibility of different individuals. At some seasons there have been epidemics of dermatitis so widespread that public schools in San Antonio, Texas, were closed until the surrounding trees could be sprayed.

The severity of the sting varies with its location and the thickness of the skin affected. There is at first a localized, painful area of erythema, which burns intensely. Small vesicles then appear; whitish

* An abstract of this article was read before the Research Club of The Harvard Medical School, December 16, 1921.

spots on the red background and slight swelling may be present in the vicinity of the lesion. Sometimes a sting upon the wrist will cause a swelling of the entire arm. There may be generalized symptoms of numbness in the part affected, or even in other limbs, with a pseudo-paralysis. Carpenters have reported that they were practically unable to grasp a hammer or to work for several hours after being stung upon the wrist or hand. In children there may be from 1-5° of fever, extreme restlessness, and often nausea and vomiting, mild general spasms sometimes following the attack. As the local symptoms subside the skin may remain discolored for some time, reddish or almost blackish areas marking the site of the lesion.

### *Megalopyge opercularis.*

The caterpillar is the larval form of the moth *Megalopyge opercularis* (Fig. 1), which has a distinctly southern distribution in this country, occurring abundantly only south of the Red River in Texas, although it is found casually in the Southern States. Other species of the genus are found in Mexico, Central and South America (thirteen species in Brazil (von Ihering, 1914)), and the West Indies. It is a comparatively small larva, measuring about 2 cm. in length and 1 cm. in width, if one counts in the soft, luxuriant coat of tawny to grayish hairs which gives it its popular names. Its color varies from dark fawn, through tawny red, to gray, and the caterpillars vary in size, some being as large as the drawing in Fig. 1, others almost a third smaller. Beneath the hairs are concealed the tubercular ridges, or verrucæ, common to many caterpillars, which bear rows of sharp, needle-like spines (modified setæ) like porcupine quills, which are hollow structures that penetrate the human skin and cause the dermatitis just mentioned. In general appearance the caterpillar so closely resembles the larva of *Lagoa crispata*, the flannel-moth, which occurs along the Atlantic Seaboard to New England and the Northern States, that the two species are practically indistinguishable to an inexperienced observer. For a detailed description of the adult moth of *Megalopyge opercularis*, of the various instars of the larva, of its cocoon, and of its habits, the reader is referred to an article by Mr. Bishopp, which is to be published shortly.

The dorsal setæ, or hairs, of *Megalopyge opercularis* are twice as long over the ninth and tenth abdominal segments as they are over the first eight, and form a fairly conspicuous tail. The fourth and fifth ocelli are close together in *Lagoa*, rather distant from each other in *Megalopyge*.

The family Megalopygidæ belongs to the suborder Frenatæ and comprises three genera: *Carama*, *Lagoa*, and *Megalopyge*. It is important from an entomological standpoint as forming one of the links in the Zygænoidea, standing between the Zygænidæ and the Cochlidiidæ (or Limacodidæ). "Its members are particularly interesting as transition forms and their structure is the clue to the peculiarities of the slug caterpillars" (Fracker, 1915). The distinguishing features of these genera are fully described by Fracker. All of their North American species are capable of inflicting more or less painful stings through the agency of poisonous spines, the anatomy of which will be described in the course of this paper.

## *Urticating Caterpillars.*

Three superfamilies of moths include practically all of the urticating caterpillars, the Zygænoidea, Bombycoidea, and Saturnoidea. In Table I the principal families are grouped under these superfamilies and a few representative genera are noted under each, with their common names. The list is by no means complete; it is meant as a suggestive tabulation, rather than a classification. Fracker's classification has been followed, which accounts for the presence of *Automeris io* under the Hemileucidæ, rather than under the Saturniidæ (Table I).

*Geographical Distribution.*—Urticating caterpillars are world-wide in their distribution, but the most offensive species appear to inhabit South America.

*Europe.*—While all three superfamilies are represented here, two genera stand out as being the most troublesome, the processionary caterpillars of the genus *Thaumetopœa* (*Cnethocampa*) and the brown-tailed moth, *Euproctis chrysorrhœa*, well known in the Eastern States of this country. The first named genus has been known from antiquity, the ancients employing it as a vesicant. Lists of the European urticating caterpillars will be found in Tyzzer's article (1907) on brown-tailed moth dermatitis and in Railliet's text-book (1895).

*Asia.*—Caterpillars of the family formerly called Bombycidæ have been known to cause trouble in Ceylon and India (Castellani and Chalmers, 1913), and Tyzzer mentions a Philippine caterpillar, *Taragama igniflua*, whose spines are somewhat similar to those of the Megalopygidæ, but slightly barbed, instead of smooth shafted.

*Africa.*—Four families are mentioned in Castellani and Chalmers' text-book as being capable of producing urtication: (1) the larva of a tiger-moth, of the

TABLE I.

| Superfamily. | Family. | Genus. | Species. | Common name. | Habitat. |
|---|---|---|---|---|---|
| Zygænoidea. | Megalopygidæ. | *Carama* | *cretata.* | | United States. |
| | | *Lagoa* | *crispata.* | Flannel-moth. | " " (Northern). |
| | | *Megalopyge* | *opercularis.* | Puss-caterpillar, etc. | " " (Southern). |
| | | " | *lanata.* | *Tâta-râna, gusano pelo de Indio.* | Brazil, Colombia, etc. |
| | | " | *albicollis.* | *Tâta-râna.* | " |
| | | " | *radiata*, etc. | " *pollo, perrito.* | " Colombia, etc. |
| Bombycoidea. | Cochlidiidæ. | *Sibine* | *stimulea.* | Saddle-back moth. | United States. |
| | (Limacodidæ). | *Natada* | *nasoni.* | | " " |
| | | (Various Brazilian genera.) | | | South America. |
| | Thaumetopœidæ. | *Thaumetopœa* | *processionea.* | Processionary caterpillars. | Europe. |
| | | (*Cnethocampa*) | *pityocampa.* | | |
| | | | *pinivora.* | | |
| | Lasiocampidæ. | *Taragama* | *igniflua.* | | Philippines. |
| | Liparidæ. | *Euproctis* | *chrysorrhœa.* | Brown-tailed moth. | Europe, United States. |
| | Arctiidæ. | *Arctia* | *caja.* | Woolly bear. | " Canada. |
| | | (Unclassified?) | | *Oruga Santa Maria.* | Colombia. |
| Saturnoidea. | Saturniidæ. | (Various Brazilian forms.) | | | South America. |
| | Hemileucidæ. | *Hemileuca* | *maia.* | Buck-moth. | United States. |
| | | *Automeris* | *io.* | Io moth. | " " |
| | | " | *cinctistriga.* | *Gusano perejil.* | Colombia, South America. |
| | | " | *viridescens.* | | Brazil, " " |

Arctiidæ; (2) a member of the Cochlidiidæ; (3) one of the Liparidæ (to which the browntail belongs); and (4) a member of the old group Bombycidæ, now subdivided into several new families.

*North America.*—The best known and most troublesome caterpillar on this continent is the larva of *Euproctis chrysorrhœa*, the brown-tailed moth. The pathology of the lesions it produces, as well as the mechanism by which they are brought about, were studied and reported by Tyzzer in 1907. A list of the native caterpillars that have urticating hairs will be found in Herrick's (1916) and Riley and Johannsen's (1915) text-books, and will be seen to contain members of all three superfamilies previously cited. Four of the more commonly encountered native caterpillars with urticating propensities are: *Lagoa crispata*, the flannel-moth, studied and reported by Packard (1894); *Hemileuca maia*, the buck-moth; *Sibine stimulea*, the saddleback; and *Automeris io*, the Io moth. The last is much sought after on account of the beauty of its imago, and is probably the best known of the four.

*South America.*—This continent, particularly its northern portion, is the habitat of many noxious caterpillars, belonging to the three superfamilies mentioned above. Roughly speaking, there are five groups of stinging caterpillars in that region: (1) those belonging to the family of Megalopygidæ (thirteen species), represented by the popular group *táta-ránas* of Brazil (Göldi, 1913; von Ihering, 1914; Bleyer, 1909); (2) those included in the Cochlidiidæ and occurring in Brazil; (3) those of the family Arctiidæ, found in Brazil and Colombia (*oruga Santa Maria* (Garcia, 1910)); (4) a group resembling our buck-moth and Io moth, and belonging to the Hemileucidæ, found in the same general regions (Göldi, Bleyer, and Garcia); and (5) a group belonging to the Saturniidæ and described by Bleyer in Brazil. These varieties are illustrated and described in the articles just cited. By far the best article on the urticating caterpillars of Brazil is that of von Ihering (1914), to which the reader is referred for further particulars concerning the *táta-ránas*. This name is from the Tupi-Guarani dialect and simply means fire-like; it is thus popularly applied to any urticating caterpillar, although it usually means one of the Megalopygidæ, the true *táta-ránas*.

The distribution of the stinging caterpillars has been outlined here at some length, because I have not been able to find it summed up in any one article thus far written.

*Lesions and Symptoms Caused by These Genera.*—The lesions produced by the sting of these genera vary from simple erythema, with burning or itching, to more extensive inflammation, with the production of papules, vesicles, or bullæ. The conjunctiva is sometimes affected by nettling hairs that retain their irritating properties after becoming detached from the caterpillars, as in the case of the processionary and brown-tailed moths. Cases of inflammation of the respiratory mucosa have been reported in connection with the former (Laudon, 1891). Goossens (1881, 1886) experimented upon himself with this caterpillar and produced very unpleasant symptoms, his entire body becoming swollen and his eyelids so edematous that he could no longer see to continue the experiment. Cases of like

gravity are described by Göldi, Bleyer, von Ihering, and Garcia, most of them occurring in connection with members of the genus *Megalopyge*. Their sting causes intense pain, swelling of an entire member, lymphadenitis, and stiffness and disability of the affected side. Bleyer describes a sphingid moth whose sting produces twitching of the affected member and even of the facial muscles on the affected side. Garcia describes symptoms of conjunctivitis and tonsillar angina following the sting of *Megalopyge lanata* (*gusano pelo de Indio*), "severe enough to be considered potentially fatal."

*Types of Nettling Hairs.*—There are two large classes of nettling hairs, those readily detachable and those that do not come off easily, but break off in the epidermis of the victim after direct contact with the insect. The latter type may be simple and quill-like, or with barbs (*Taragama*); or they may be branched like small trees. Various types are shown in Fig. 6, from Göldi's article. The detachable type is represented by the nettling hair of the brown-tailed moth and processionary caterpillars, which is dangerous after an entire season, following detachment from the insect. The smooth, spinous type with which we are concerned in this paper is described by Packard (1894, 1898) in connection with *Lagoa crispata*. He believed the venomous fluid to be contained in the spines and to be secreted by several large cells at their base. Von Ihering (1914) describes an almost identical apparatus for an unspecified species of *Megalopyge* which he studied in São Paulo, Brazil. Leydig describes "poison" oozing through a pore in the tip of such a seta, but Berlese thinks this erroneous and believes that the venom leaves the shaft by osmosis through the chitinous wall. Discussing this question, von Ihering says that he has thought on several occasions that he could demonstrate a subapical pore in these spines, similar to that in a serpent's fang. He also says: "Upon irritating the animal, it erected its hairs in the usual manner and we then saw a little drop of liquid exude from the tip of each of the spines which serve to defend the caterpillar." He goes on to point out that, should the spine be broken off in the skin of the victim, it would be useless for further action. As will be seen later this is not necessarily true, for the venom dries and forms a pigmented, gummy plug that effectually seals the broken extremity.

*Nature of the Venom.*—As to the nature of the poisonous substance, very little is known. Goossens believed that he had isolated cantharidin from the hairs of the processionary caterpillar; but Laudon disproved this, substituting the conjecture that it is formic acid, a supposition that has held with considerable tenacity up to the present, although it is obvious that this acid alone cannot cause the symptoms produced by the sting of these caterpillars. Bleyer noted a strong odor of this acid in broken spines from a sphingid caterpillar but tests with litmus proved the substance to be alkaline. Tyzzer experimented upon the solubility of the venom of the browntail caterpillar, as well as with its reaction to heat, chemicals, etc., but came to no definite conclusion as to its chemical composition. He found that it had a tendency to break up the rouleaux of human erythrocytes and to shrink the latter after a brief phase of crenation. The venom is present in the spines in such minute quantities that an analysis is extremely difficult.

*Original Work on Megalopyge opercularis.*

*Production of Gross Lesions.*—The larvæ used in these experiments were shipped from Texas in mailing-tubes; some of them, however, had spun cocoons in transit and were used in their prepupal form. The larva was rubbed over the slightly moistened skin of the bend of the elbow, anteriorly in four individuals. The prickling of the tiny spines was distinctly felt in each case. Next a burning sensation was experienced, which began almost immediately in the individuals with thinner skin, and appeared a minute or so later in those with thicker skin. This persisted from $\frac{1}{2}$ hour in one case to 3 to 5 hours in the others. Shortly after the onset of burning, several small papules about 2 mm. in diameter, with a yellowish red cast, appeared over the surface of the erythematous area. The latter attained a diameter of about 4 cm., but the concentric rings of red and white, described by Mr. Bishopp as encircling it, were not observed in any of these four experimental cases. After the erythema subsided the papules persisted for about 48 hours to 3 days; they formed no large vesicles and itched in only one case out of the four. There was a suggestion of slight hemorrhage in their vicinity, giving them a petechial appearance.

A second application, 2 days later, in the case of the writer, produced more marked symptoms and slight itching. A third, about 5 days after the first, was carried out on both forearms in multiple, several caterpillars being tested out for other phases of the experiment. In this case the reaction was somewhat different; the erythema was less painful, but no less intense, and was followed by the production of wheals of an average diameter of about 2 cm., which itched intensely. These had small yellowish, slightly vesicular papules superimposed upon them and persisted for 3 days, after which the itching and swelling gradually subsided. Small reddish papules, with tiny scabs at their apices, were still present after a week. One of the lesions was excised for examination at the height of the inflammation, after 24 hours development, by plunging a skin hook into its center and clipping off an ellipse of skin 1 by 0.5 cm. in size, with straight scissors. This was done without anesthesia, in order to avoid distorting the lesion. The wound healed uneventfully, the caterpillar venom causing no complications. During the develop-

ment of the multiple wheals the forearm became hot, itched intolerably, and there was a sensation of dull pain extending distally to the wrist; but no paralytic or generalized symptoms were noted. The prepupal stage of the larva caused similar, but slightly less intense symptoms, whereas a dried, dead larva was only urticating when vigorously rubbed in. Apparently the toxicity diminishes somewhat after the death of the caterpillar.

Although it was almost certain that the setæ, or spines of the tubercles, were the cause of the reaction, the other hairs, which are long and feathery, were cut off and rubbed into the moistened skin; merely a slight scratching was produced. Juices of the skin and body cavity of the caterpillars were inoculated into small wounds made by cross-hatching the skin with a sterile sewing needle. Although the juices of the brown-tailed caterpillar were found to be very, and almost immediately toxic by Tyzzer, those of *Megalopyge opercularis* produced no reaction. This coincides with the findings of von Ihering, who experimented with inoculated body fluid of a *Megalopyge* and produced no lesion, although the venom from its setæ promptly caused a typical reaction.

Next a verruca was cut off the caterpillar's skin and held in fine forceps so that its setæ were presented to the skin of the subject like the bristles of a brush. Brisk rubbing gave results precisely like those obtained by rubbing the entire larva over the integument; the venom is, therefore, contained in these spines and limited to the verrucæ. In order to test for the presence of venom in the poison sacs and hypodermal glands (which will be described later), a caterpillar was pinned down on a board and eviscerated. The inner surface of the skin was sponged clean with cotton, scraped with a scalpel and the scrapings were rubbed into scarified areas on the human skin. Then, after scraping as much as possible, without tearing, a small square of the caterpillar's skin was excised and placed with its inner surface closely apposed to a second scarified area on the writer's arm. Both experiments produced positive results, although a control scarification showed no such change. Setæ pulled out and held base downward were rubbed into an excoriated area; this also produced very slight, but positive reactions. These three experiments show that the venom is contained in the cuticle of the caterpillar and in the base of the setæ as well as in the hollow shaft.

Rabbits' ears rubbed over the inner surface with the larvæ become reddened and swollen and then droop for about 24 hours; but they show no wheals, vesicles, or papules. A caterpillar rubbed over the shaven abdomen of a mouse produces almost immediate symptoms, the mouse scratching and licking the inoculated area for about ½ hour. Tiny vesicles and a skin reaction similar to that seen in the human subject were apparent; under the microscope the lesions showed a simple leucocytic infiltration.

*Treatment.*—The dermatitis responds rather readily to any soothing lotion or antipruritic; the proprietary Obtundia Cream was found especially soothing, although carbolated vaseline was also effective. If the lesions become excoriated, compound tincture of benzoin or tincture of iodine is indicated. The immediate application of an alkali, such as ammonia, is without any beneficial effect and is not to be recommended; in spite of it the erythema and vesicle formation proceed unabated.

*Anatomy of the Urticating Apparatus.*—The anatomy of the stinging apparatus is comparatively simple. A number of transverse tubercular ridges, or thickenings, are distributed over the surface of the cuticle of each segment. Roughly speaking they are laid down in six parallel longitudinal rows over the dorsum and sides of the various segments. These are the verrucæ, whose arrangement, as well as that of the setæ they bear, is utilized by entomologists in classifying the insects. The exact arrangement of these verrucæ and setæ is given by Fracker, The tubercles consist of weakly chitinized thickenings of the cuticle, upon which are rows of bristle-like spines and tufts of feathery hairs. The former are firmly, the latter rather lightly attached, so that if the larvæ be scraped, these hairs, rather than the spines, come away. The latter are best seen in the prepupal form of the moth, for the hairs having been utilized in spinning its cocoon, the insect is comparatively naked, the verrucæ standing out clearly as bristling ridges (right hand drawing in Fig. 1). They have the appearance of minute pincushions, the spines radiating somewhat from their convexity; their grayish color and blackish tips stand out conspicuously.

If a verruca be excised and examined under the microscope (Figs. 2 and 3), it will be found that these setæ are slender, acute at one end, and attached to a conical or rounded projection of the verruca by a cup-shaped expansion at the base. They are of a yellowish brown color, hollow throughout, and contain masses of amorphous, dark brown pigment scattered through their lumina. They are frequently

found with broken tips, in which case a plug of pigment projects slightly from the fractured extremity. They vary in size from 0.3 to 1.0 mm. in length and from 15 to 45$\mu$ in diameter. Apparently most of them are broken at the tips, but the smaller forms are found to taper to a needle-point that does not seem to be canalized, although the lumen runs down very nearly to the apex. Tests show them to be composed of heavily chitinized material, the pigment being resistant to solvents.

*Microscopic Anatomy.*—Larvæ and prepupal larvæ were fixed in Zenker's fluid, cut to 5 $\mu$ in paraffin, and stained with hematoxylin-eosin, eosin-methylene blue, and phosphotungstic acid-hematoxylin. Microscopic examination shows that the cuticle is thickened to form the verrucæ (Fig. 2) and, instead of being solid and laminated as elsewhere, is filled with cells resembling those of the hypodermal layer. In fixed material these cells shrink away from the cuticle, lying in globular cavities; but unfixed material does not show any unfilled spaces. As the surface is approached one finds the cells increasing in numbers until, in the upper layers, they almost touch one another. The surface layer shows poison setæ and ornamental hairs alternating with one another. Beneath the verrucæ there is a heaping up of the hypodermal layer of cells to form gland-like organs, whose ducts spiral outward somewhat like those of the human sweat glands and communicate with the hair roots. As these readily can be demonstrated to connect with the roots of the feathery hairs, but only occasionally are seen to lead into that of a poison seta, it is questionable whether they are still functioning in this case; they may have transmitted the venom to the poison sac of the seta at an earlier data and then atrophied or they may be columns of setigerous cells, as interpreted by Packard (1894) in connection with *Lagoa crispata*. According to von Ihering's interpretation, they are at first trichogenous, forming the seta, and then become toxogenous, secreting the poison that is stored in the theca, or poison sac. As they appear to be atrophic in the stage examined here, although the setæ are highly venomous, this point will have to be decided by a careful study of the setæ in various instars of the caterpillar.

The structure of the poison setæ is best seen in the smaller and medium sized forms of the larger tubercles. Fig. 4 shows one of these

diagrammatically, the drawing being a reconstruction from serial sections. The shaft (*Seta*) is hollow and broken off at its tip, which is plugged with a mass of pigment (*Pigm.*) The base expands into a chitinous collar, continuous with the shaft, which embraces a conical projection of the cuticle (*Coll.*), forming an inner collar. Just above this is a chitinous diaphragm (*Diaphr.*) pierced by a small opening, or fenestra. The cuticle forms a shell (*Bulb*) for the internal structures of the organ and usually shows one or two nuclei at its base (*Bulb n.*), which may belong either to the setal root, or to the theca, or poison sac, though the latter seems unlikely, as they differ somewhat in appearance from those of the sac. At the base of the cuticular shell is a carrot-shaped structure, staining darkly with phosphotungstic acid-hematoxylin and with eosin, which appears to connect with the remnant of one of the spiral ducts or cell columns in some instances. This is not universally present and is found in a small proportion of the setal bulbs. It is similar to the root of the feathery hairs in its appearance, situation, and staining properties, and is possibly a vestigial root (*V. r.*).

The theca, or poison sac, so called because of its anatomical continuity with the duct that traverses the setal shaft, although it cannot yet be demonstrated that its contents are nothing but the venom, is a thin walled bulb with several delicate, flattened, elliptical nuclei sparingly distributed over its surface (Fig. 4, *P. s.*; Fig. 5). It communicates with a slender tube, or duct (*P.d.*), constricted as it traverses the fenestra in the diaphragm and expanding slightly to terminate somewhere near the tip of the shaft, where it thins out and becomes difficult to follow. Nuclei are scattered over its surface and are best seen in phosphotungstic acid-hematoxylin sections. No muscular tissue is demonstrable in connection with the sac or duct. Both of them are filled with a continuous mass of finely granular, fixable substance in which bubble-like vacuoles are seen occasionally. The wall of the sac, although it contains several nuclei, shows no cell partitions in the sections examined; it appears to be syncytial, though silver nitrate preparations might demonstrate cell boundaries. Sometimes a delicate membranous partition appears to bisect the sac in a plane at right angles to its long axis.

These basal organs of the setæ vary somewhat in respect to shape and location; they may be as represented in the figures, or constricted at their middle to form a figure of eight. They may lie embedded in the cuticle, with the collar of the shaft flush with the surface, or they may project so far above the surrounding cuticle as to be constricted at their base to a degree that renders them pedunculated. Such setæ are more readily detached than their more deep seated neighbors.

The poison apparatus of this caterpillar corresponds in every particular with that of *Lagoa crispata* as described by Packard (1894, 1898) and of the *Megalopyge* described by von Ihering (1914), which is to be expected in the case of such closely allied genera; it is totally different from the unicellular apparatus of the genera described by Holmgren.

*Pathology of the Lesions in Human Skin.*—The lesion excised from the arm was dropped immediately into Zenker's fluid and, after fixation, cut into some 280 practically serial sections at 7.5 $\mu$. They were stained in eosin-methylene blue and phosphotungstic acid-hematoxylin. There are approximately ten fragments of setæ found embedded in the epidermis of the lesion; none of them penetrates to to the corium, and yet the latter shows marked pathological changes. The seta enters the epidermis by pushing between cells and forcing them into a compact, encircling layer in its immediate vicinity (Figs. 7 and 8). Where the setal shaft is intact this is the only effect. Some of the setal fragments represent the tip broken off in the epidermis, others portions of the shaft that apparently have lost their tips before penetrating the skin, like the extremity of that in Fig. 4. Where the lumen of the shaft communicates with the surrounding epidermis, the cells of the latter are killed or dissolved (Figs. 7 and 8). A coagulum similar to that found in the venom sac and seta of the insect can be seen in the shaft fragment, oozing out into the cavity left by the destruction of the epithelium. Sometimes the necrosed epithelium forms a denser and more darkly stained coagulum. The loss of cells about the setal fragment is comparatively extensive, and vesicular cysts constitute the typical lesion (Figs. 9 and 10). These lie in the epidermis and may or may not show setal fragments in their walls, according to the section. They usually contain short threads of fibrin in a clear space and are invaded by a moderate number

of lymphocytes in their more advanced stages. They measure from 150 to 350 $\mu$ in diameter, apparently varying directly in proportion to the size of the seta that caused them. The usual diameter is 150 $\mu$ and is remarkably constant. The largest lesion (Fig. 10) was found to lie beneath a large puncture wound, shown in the photograph, filled with coagulum which showed small vacuoles. The puncture measures 30 $\mu$ in diameter, the size of one of the larger setæ; the latter may have entered the skin and been withdrawn, leaving its venom behind, for no remnants of the seta can be found. The average diameter of the setal fragments found in the skin is from 14 to 18 $\mu$, therefore the ratio of the diameter of the lesions caused by the smaller setæ to that of those caused by the larger is approximately the same as the ratio between the setal diameters.

Above the vesicles the keratinized layer of the epidermis is raised to form a small blister, filled with granular coagulum, but this is not always the case. Occasionally a small scab of coagulated and necrosed epidermis surmounts them; this is the tiny scab that persists after the lesions have subsided. Below the vesicular, primary epidermal lesion, the cells of the prickle and germinative layers are separated and degenerated and the tissue is edematous. Almost every blood vessel in the edematous corium is surrounded by an exudate of lymphocytes, eosinophils, and endothelial and polymorphonuclear leucocytes (Fig. 11). The lymphocytes outnumber the other cells very noticeably, the eosinophils are next in order of frequency, and the endothelial and polymorphonuclear leucocytes about equally represented and not very numerous. The lumina of the capillaries show many lymphocytes and eosinophils, and those of the lymphatic system are sometimes almost clogged with the former. The vascular endothelium is not appreciably swollen, nor are its cells increased in number, no mitoses being found in them.

The reaction is, therefore, of the type that Tyzzer describes in connection with the brown-tailed moth; the similarity is at once apparent if one compare the illustrations of this article with his. It is a deeper and more destructive lesion than is that of the browntail and is essentially what one would interpret as a reaction intended to neutralize a poison, as lymphocytes are the most abundant cells in the perivascular exudates. Eosinophils, so commonly seen in skin reac-

tions in connection with cellular decomposition, are second in order of frequency. These leucocytes also respond to the secretions of animal parasites and to vegetable proteins (*cf.* asthma and hay fever); the character of the reaction, therefore, points to a protein irritant.

*Nature of the Poison of Megalopyge opercularis.*—Boiling a caterpillar in water for a few minutes, after killing it, renders its venom inert; after drying, the spines may be rubbed into the skin with impunity. An hour in water at 55°C. has no effect upon the venom, but this temperature will have the same effect as boiling if the caterpillar be left in water at 55° over night. It is impossible to extract enough venom from the setæ of a few caterpillars in a little water or salt solution to get any definite chemical reactions. Such extracts are also unsatisfactory for testing out on the skin.

On the other hand, it is possible to obtain the venom in another way. By extracting the ground skin and hairs of these larvæ and their prepupal form with distilled water, or salt solution, an impure mixture of extractives is obtained. This contains the venom and causes symptoms when inoculated into the human skin, or injected into animals. A detailed description of the work done in this way will be reserved for another paper. It will be enough to state here that these extracts cause the same reactions in the human skin (burning, erythema, and itching, with the production of wheals and vesicles) as do the caterpillars themselves. Injected intravenously into rabbits they produce pupillary contraction, conjunctival edema, restlessness, and convulsions and death when administered in sufficiently large doses. Given intraperitoneally to mice they cause roughening of the coat, profuse defecation, with retraction of the flanks, stiffened and uncertain gait, and death in an hour following the administration of 10 mg. of crude desiccated extract in 0.5 cc. of normal salt solution. When applied in small quantities to isolated strips of cat smooth muscle *in vitro*, they intensify the rhythmic contractions and slow down their rate. This effect is almost immediate and produces exhaustion and death of the strip after 15 minutes or so.

## CONCLUSIONS.

1. The dermatitis caused by contact with the larva of *Megalopyge opercularis* is produced by a poison introduced by the hollow,

specialized setæ of its cuticular tubercles. It is not produced by the ornamental hairs, or by the tissue juices of the animal.

2. The poison appears to be of the nature of a venom, combined with protein vehicles, and may be itself a protein.

3. It is rendered inert by boiling, or by heating to 55°C. for a considerable period of time.

4. It is apparently stored in sacs at the base of the setæ, but whether secreted there, or by hypodermal glands, remains to be determined.

5. It diminishes in virulence after the larva has spun its cocoon, and is no longer active after the caterpillar is dead.

6. The poisonous spines cause localized necrosis of the human epidermis, followed by the formation of small vesicles. The cellular reaction to the poison is chiefly lymphocytic.

The writer is very grateful to Mr. F. C. Bishopp, of the United States Bureau of Entomology, for the opportunity of studying this caterpillar, for the caterpillars furnished, and for his advice and information. So many individuals from the Bussey Institution, the Smithsonian Institution, and various departments of the Harvard Medical School have given me advice and help that it is impracticable to list their names; their assistance is none the less appreciated.

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## EXPLANATION OF PLATES.

### PLATE 62.

FIG. 1. Pencil drawing of the extended and contracted larva of *Megalopyge opercularis* and of the prepupal stage of the caterpillar (head downwards). Note the rows of blackish setæ in the prepupal phase and the comparative absence of long hairs. Slightly enlarged.

FIG. 2. Section of a verruca. This shows the thick cuticle with a row of setal bulbs on its surface, two of them with setæ attached. Note the carrot-like root of an ornamental hair near the middle of the row, with its spiral-shaped lower extremity. The barbed hairs are the non-stinging, ornamental type. In the body of the verruca is the gland-like thickening of the hypodermis, which may be connected with the secretion of venom. Eosin-methylene blue. × 100.

FIG. 3. Unstained setæ attached to a verruca which has been cut off; at the left a detached seta with its theca and diaphragm is clearly visible. Note the pigment in the shafts, their double lined contour, and the absence of tips from several setæ. The black spots in the verruca are the roots of ornamental hairs, some shafts of which are dimly seen at the right, among the setæ. × 100.

FIG. 4. Schematic diagram of a seta. *Pigm.*, pigment plug; *Seta*, setal shaft; *P. d.*, poison duct; *Diaphr.*, diaphragm of the shaft; *Coll.*, collar of the seta; *P. s.*, poison sac, or theca; *Bulb*, cutaneous bulb containing the poison sac; *Bulb n.*; setigerous (?) nuclei; *V. r.*, vestigial root of the seta. (*Cf.* hair roots in other figures.) × 250.

FIG. 5. Setal bulb and part of shaft, with diaphragm. Note the thickened collar, the hair root at the left of the bulb, the nuclei of the poison sac, and the small calcareous granules. Phosphotungstic acid-hematoxylin. × 450.

### PLATE 63.

FIG. 6. Urticating hairs of various European caterpillars, from Göldi, after Lampert. (*a*) "*Kupferglucke*," *Gastropacha quercifolia;* (*b*) nun-moth, *Ocneria monacha;* (*c*) another type of hair from *Gastropacha quercifolia;* (*d*) woolly-bear, *Arctia caja;* (*e*) the peacock, *Vanessa io;* (*f*) European tussock-moth, *Orgyia antiqua.*

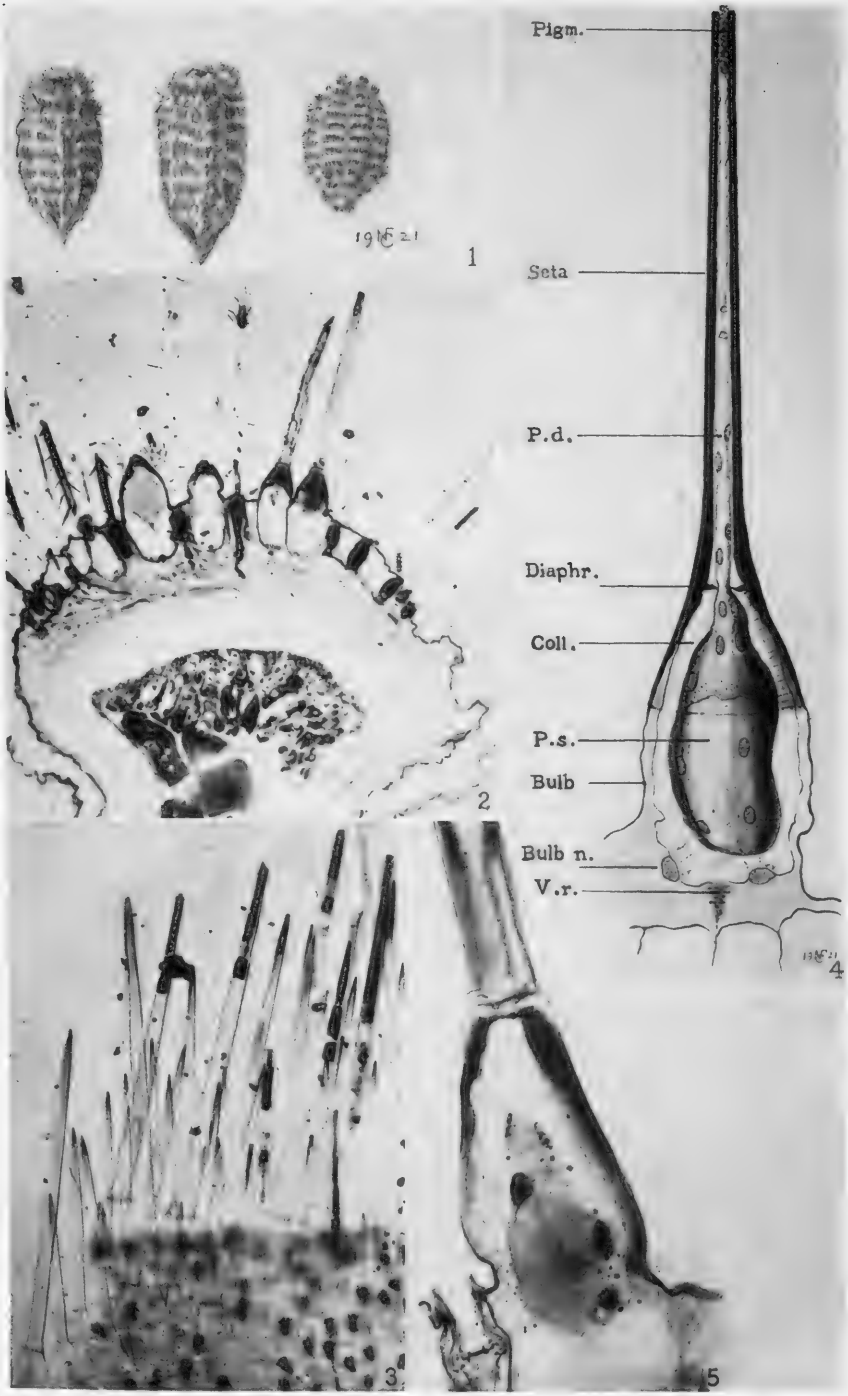
FIG. 7. Setal fragment in human epidermis. The coagulum about the fragment is chiefly cellular debris from the epithelium. The fragment is blunt at both ends and probably represents the tip of a seta that had been previously fractured at its point. Note the pycnosis of the neighboring nuclei and the beginning vesicle. Eosin-methylene blue. × 450.

FIG. 8. Setal tip in a papular lesion, with a vesicle forming at its point and to the left. The compression of the epithelium in the immediate vicinity of the seta and the degenerated condition of its cells are quite marked. There are coagula at the point of entrance, which forms the apex of the papule, and at the tip of the seta. Phosphotungstic acid-hematoxylin. × 450.

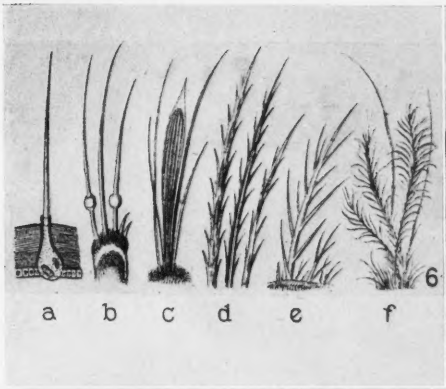
FIG. 9. Large vesicle to the left of a setal fragment, which is seen in the superficies of the right wall, surmounted by a small, detached scab. Phosphotungstic acid-hematoxylin. × 100.

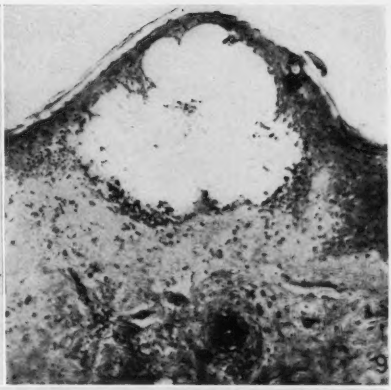
FIG. 10. Section of another vesicle. The puncture wound referred to in the text is seen at the apex, plugged with coagulum. Strands of fibrin and lymphocytes are present in the vesicle, and the infiltration of the corium by the lymphocytes is well shown. Note the perivascular infiltration below, in the right hand corner. Phosphotungstic acid-hematoxylin. × 100.

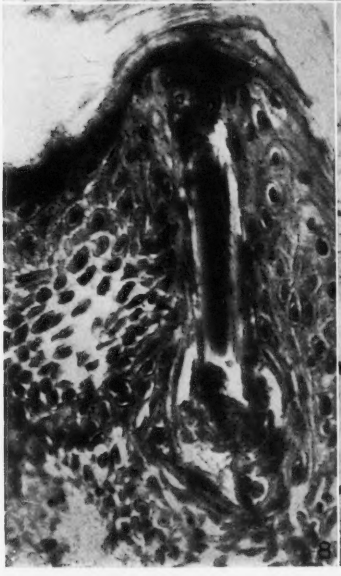
FIG. 11. Perivascular lymphocytic infiltration and distended lymphatic with fibrinous coagulum. Eosin-methylene blue. × 450.

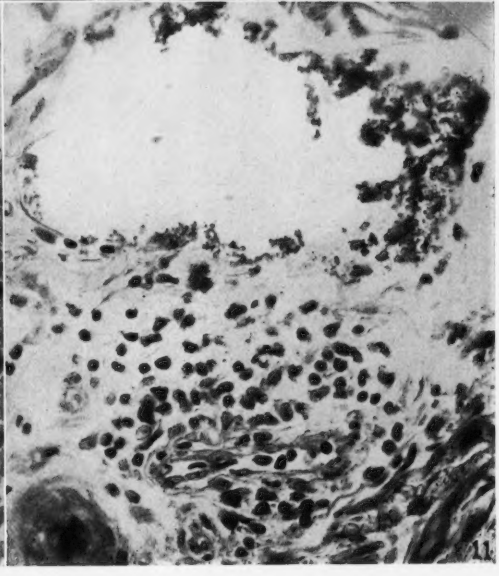



(Foot: *Megalopyge opercularis*)






(Foot: *Megaiopyge opercularis.*)